BIOCHEMISTRY OF NITRIFICATION IN SOIL

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Despite its great importance in the field of agriculture and despite the intriguing question of the intermediary metabolism involved, the process of nitrification has received relatively little attention and that only spasmodically. When Pasteur demonstrated in 1862 the microbiological nature of the oxidation of alcohol to acetic acid, he suggested that the oxidation of ammonia might have a similar origin. In 1877 this suggestion was verified (59) and for the next ten years the process received attention, culminating in 1891 in the isolation of the responsible organisms (21, 66, 69). The next advance did not come until 1916 when Meyerhof (42) undertook an extensive investigation of the respiratory activity of these organisms and studied also the effects of inhibitors. A variety of reports on the subject of nitrification made their appearance in the next thirty years but no serious attempt to study the metabolic activities of these organisms was made until the advent of the soil perfusion apparatus (35) in 1944. Since then several reports of results obtained using this technique have been published and they obviously indicate a renewed interest in the subject of soil nitrification and in particular in the biochemical mechanisms involved.

When, in 1877, it was first shown (59) that the oxidation of ammonia, under soil conditions, was probably biological, the method used was not unlike the modern perfusion technique. A column of sterile sand and chalk one meter long had sewage poured over it daily. Analysis of the sewage and the effluent were made each day and after twenty days the ammonia nitrogen input was found to appear in the effluent almost completely as nitrate nitrogen. The process was carried on for as long as four months. It was shown to be inhibited by antiseptics such as chloroform, and the activity was restored after thorough washing with sterile water and subsequent addition of washings from ordinary garden soil. The technique showed that none of the organisms then known was able to oxidize ammonia under these conditions, that alkaline conditions assisted nitrification, that oxygen was essential and that nitrite often appeared in the effluent.

During the next decade there were many attempts to isolate the responsible organisms using the then recently discovered "solid media." All these, however, failed and it was not until 1890–1891 that three independent workers (21, 66, 69) were able to isolate the nitrite oxidizing organism. Winogradsky isolated also the nitrite forming bacteria and his method of using silica gel instead of the standard organic media is still in use today (41). From these results it was established that the oxidation of ammonia proceeded in two stages:

$$NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$$

The fact that the presence of organic matter in the medium inhibited completely the growth of the nitrifying organisms led the early workers to assume that organic matter *per se* was deleterious to the growth of these organisms. Since the later nineteenth century this assumption has been the subject of much discussion but comparatively little experiment. Some idea of the course of this discussion may be obtained from a few of the papers (8, 9, 12, 17, 23, 25, 27, 29, 34, 37, 42, 51, 53, 58, 61, 62, 68, 70, 71) on the subject and from some of the reviews (6, 34, 37, 67). Later work divides organic inhibitors into two broad categories, i.e., those that produce a specific inhibitory action on the nitrifying organisms at concentrations of 0.01 M or less, and those that produce inhibitions only at very high concentrations. For example it has recently been shown (27) that a species of *Nitrosomonas* isolated from farmyard manure can oxidize ammonia in the presence of 10% glucose (0.56 M) or 20% sucrose (0.58 M), higher concentrations are inhibitory. Several examples of specific inhibitory actions will be discussed in the experimental sections of this article.

Early results led to some discussion of the mechanism of the oxidation of ammonia, but even today the details of this mechanism remain obscure. The question of the organic intermediary metabolism of the autotrophs is of obvious importance but until now has received little attention. A claim has been made that the formaldehyde detected in the growth of Nitrosomonas represents an intermediate in the reductive fixation of carbon dioxide (30).

Some account may be given at this stage of the behavior of nitrifying organisms in soil, their natural habitat. The first significant difference between their behavior in soil and in pure culture is their response to the effect of organic matter. As early as 1904 it was shown (68) that peptone is far less inhibitory in sand than in solution cultures, and in 1910 it was shown (62) that in soil, itself, nitrification is less inhibited than in silica gel media by the presence of organic matter in general. In 1909 the nitrifications of both cottonseed meal and ammonium sulphate were compared (61) in soil and solution; both were nitrified more rapidly in soil. It has also been shown (3) that soil, even after ignition, is a far better medium for supporting nitrification than is sand. Similarly cinders are more effective than porcelain or pumice as a medium and compared with these, sand and brick are inert (11). Symbiotic development of heterotrophs, which is said to play a part in nitrification in soil, would, if present, greatly influence this process (18, 47). Winogradsky himself pointed out in 1931 (70) that the data concerning the behavior of the nitrifying organisms in pure culture had still to be applied to the development of these bacteria in soil. The optimum temperature for nitrification is dependent on the site of isolation of the organisms (64) but in the temperate regions it is approximately 25 C. Nitrification may be observed (13, 40, 42) over a wide range of pH [5–13], but the actual range with an isolated organism seems to be dependent on the pH of the soil from which it was isolated. Nonbiological nitrification has also been discussed (19, 50, 63).

A number of workers (2, 15, 72) have recently shown that the presence of colloids in culture media can influence bacterial behavior, and doubtless some

similar effect occurs in soil. Another factor to be considered in soil nitrification is the effect of variation in strain of the bacteria; for example, it has been shown (13, 22, 48) that bacteria isolated from different soils have different nitrifying capacities.

It is to be expected that in soil, with its complex microflora and its special physico-chemical conditions, the kinetics, possibly even the mechanism, of nitrification, will differ materially from what takes place in pure cultures of the nitrifiers. There can be no question of the great importance of studies of pure cultures of these organisms, especially for the investigation of intermediate metabolic products which are unstable in presence of a medium such as soil. The occurrence of hyponitrous acid during nitrification, for example, in flask culture has been demonstrated (7, 16) but there is as yet no clear evidence of the formation of this substance during nitrification in soil. Hydroxylamine has long been suggested (31) as a potential intermediate in the oxidation of ammonia but no conclusive proof of its presence has been reported. Recently, however, it has been shown (51, 53) that combined hydroxylamine may be oxidized directly to nitrite. Meyerhof's early studies (42) on the respiratory activities of the nitrifying bacteria throw light on many factors influencing the metabolism of these organisms, and have to be borne in mind in the interpretation of the phenomena of soil nitrification. But for the study of the process of nitrification in soil, it is essential to study the course of the events taking place in the soil itself and to look upon the data obtained from the study of pure cultures as complementary to those found in the natural medium.

There is now an immense literature concerning field and pot experiments to show the effects of the addition of nitrogenous substances to soil. The literature on the nitrification of organic material has been fully reviewed by Whiting (67) and has since greatly increased. Whiting considered the rapidity of nitrification of the various types of nitrogen (water-soluble, easily hydrolyzable, total) and the influence on this of the carbon content of the nitrogen compounds (see also 46). Although this knowledge can be made to be of considerable value to agriculturists who wish to know how quickly fertilizers will supply available nitrogen, it does not go far in elucidating the processes by which the compounds are, in fact, nitrified.

The effect of organic matter on the nitrifiers is still undecided. Winogradsky (70) continued to maintain that they were strict autotrophs inhibited by organic matter. The general relationship of the results obtained with culture experiments to those obtained in soil is obscure. Albrecht and McCalla (1) summarized the position in 1937 thus:

> "The conditions controlling nitrification in aqueous solution have been studied very specifically. Less definite controls and less refinement in methods have obtained for studies of this process within the soil. The complexity of a sand, silt and clay mixture, as soil, prohibits an accuracy great enough to encompass all the various chemical aspects of so delicate a process as nitrification."

Before describing the new methods and results which seem to deny this foreboding, an account of the work of Meyerhof (42) on the effects of various in-

hibitors may be given. His first two papers deal with the oxygen uptake of nitrite oxidizing organisms and although some treatment of the thermodynamic aspects of the oxidation of nitrite to nitrate and the simultaneous uptake of carbon to form organic matter is given, the larger part of the papers is devoted to the effects of related organic compounds and other inorganic ions on the process.

The only inorganic sodium salts found to give any significant degree of inhibition at concentrations of 0.22–0.15 N were sodium nitrite, iodide and borate. Most sodium salts of organic acids (0.3 N) inhibited the oxidation of 0.05 to 0.10% nitrite by 50 to 70%. In these experiments proliferating cultures were used and the inhibitions were measured by estimating the decreases in rates of oxygen uptake. Some determinations were made, however, with centrifuged bacteria. Potassium cobaltinitrite and its amminated derivatives were found to be the only complex nitrites to yield an increase in oxygen uptake, but they also caused some inhibition of nitrite oxidation. The same was true of nitrosodimethylamine; and increasing concentrations of amyl nitrite caused increasing inhibitions of the oxidation of 1% sodium nitrite.

The esters of carbamic acid showed increasing inhibitory power with increasing size of the aliphatic radicle, e.g., methyl urethane inhibited oxygen uptake by 50% at a concentration of 300×10^{-6} M but isoamyl urethane caused a similar inhibition at only 22×10^{-6} M.

Inhibition by ammonium sulphate (0.001 N) was only 8% at pH 7.5 to 8.0 but 55% at pH 9.0 to 9.5, and this was true for most of the aliphatic amines, e.g., at pH 9.3 triamylamine at a concentration of 0.00005 M caused 46% inhibition. Aromatic amines and some of the alkaloids also caused inhibition. The alkaline and rare earth metal ions caused some inhibition but mercuric and silver ions had a marked action.

The third paper by Meyerhof deals with proliferating ammonia oxidizing organisms. In this paper the effects of ammonium ion concentration, pH and oxygen tension are discussed. Thus it appeared that these organisms were most active in the presence of 0.005 M substrate, at pH 8.5 to 8.8 and at a high oxygen tension. Some evaluation was made of the effects of ions, e.g., 0.1 N sodium chloride, sulphate and nitrate produced inhibitions of 6, 23 and 18% whereas the corresponding magnesium salts produced 18, 8 and 15% inhibitions. The heavy metal ions, on the other hand, were far more toxic to pure cultures, e.g., 0.005 M Pb^{++}, Zn^{++}, Mn^{++}, Co^{++}, Cu^{++}, Ni^{++}, Hg^{++} and Ag^{+} yielded inhibitions, all of which were greater than 50%.

Organic salts of sodium gave results which were much the same as those obtained with sodium chloride. Sodium benzoate was however exceptional giving 52% inhibition at a concentration of 0.026 M (compare 0.2 M sodium chloride giving 45%).

Guanidine was found to be a potent inhibitor of ammonia oxidation and the concentrations of some of its substituted products which yield 50% inhibition were found to be as follows: guanidine 50×10^{-6} M, aminoguanidine 65×10^{-6} M, triphenylguanidine 250×10^{-6} M, nitroguanidine 19×10^{-3} M, creatine 10×10^{-3} M, creatinine 20×10^{-3} M.

Aliphatic amines or diamines, and aniline or substituted anilines, all gave inhibitory effects, e.g., *p*-phenylenediamine inhibited respiration in pure culture 100% at a concentration of 50×10^{-6} M. The simple carbamates were tested and it was found that the smaller the ester radicle the greater was its efficiency in inhibiting the respiration of the ammonia oxidizing cells.

A great deal of experimental work has been carried out on nitrification in soil itself but this has had reference mainly to problems of soil fertility. Little light was thrown on the mechanism of nitrifying processes in soil and work with pure cultures proved to be of little help. A need for a new approach to the problems of soil metabolism was obvious. In 1944 the first report of such an approach was made (35) and its main contribution was the description of a new apparatus designed to study perfused soil. Since then the apparatus has been simplified (4, 33) and it is the simplified versions which are in use today. Several reports (5, 14, 24, 32, 34, 35, 36, 37, 38, 51, 52, 53, 60) on the use of this type of apparatus have already appeared and it seems that the soil perfusion technique fulfills many of the requirements required in a fresh approach to the problems of nitrification.

The technique is such that the soil is treated throughout the experiments as a biological whole. The metabolic events taking place may be studied with greater accuracy than has been accomplished hitherto. In essence, an attempt is made to study the metabolism of a soil as though it were a living tissue. Emphasis is placed on the changes brought about by the soil as a whole under defined experimental conditions, and care is taken that the soil itself is not interfered with throughout the experimental period. It is, of course, certain that many biological and chemical changes occur in the soil during the experiment; but so long as the technique employed gives accurately reproducible results, there is no *a priori* reason why various aspects of metabolism should not be as amenable to study in soil as they are in isolated plant or animal tissues. Experiments which will be described show with what reproducibility the kinetics of these processes may be studied even in soils of different origins.

The first studies on the process of nitrification (37), using the soil perfusion apparatus, confirmed it as a comparatively slow process accomplished entirely by microorganisms. Further experiments gave rise to the conclusion that the rate of nitrification of a given quantity of ammonium sulphate is a function of the degree to which the ammonium ions are adsorbed on, or combined in, the soil in the form of the soil's base-exchange complexes. The greater the amount of adsorption, the faster is the nitrification.

The interpretation of these results was that the nitrifying bacteria grow on the surfaces of the soil crumbs at the sites where ammonium ions are held in base-exchange combination, and proliferate at the expense of such adsorbed ammonium ions.

These facts led to the conclusion, that, when all the relevant sites at the surface have been occupied, further growth of the organisms will not occur except to replace cells which have died and disintegrated. Remarkably few living nitrifying cells enter into the soil solution. There arose, therefore, the conception

of a bacteria-saturated soil; that is to say, a soil where the area of proliferation is limited and cannot be extended owing to full occupancy of available sites for proliferation. Such a bacteria-saturated soil may be made to yield information as to whether any given substance is broken down by the cells which saturate the soil. If an organic nitrogen compound, for example, is broken down and oxidized to nitrate by nitrifying cells, then the course of nitrate formation in a soil saturated with such cells should be linear, and show no initial lag period. If a lag phase does take place, the inference is that the compound in question needs attack by organisms other than the nitrifiers before nitrification can take place. In this way it has been proved that aliphatic amines, which are nitrified in soil, require organisms other than the nitrifiers to effect the initial decomposition.

An interesting new observation has been that, although hydroxylamine, at small concentrations, is toxic to the nitrifying organisms and is apparently not nitrified, the presence of sodium pyruvate secures a speedy nitrification of the amine. Pyruvic oxime also undergoes rapid nitrification by a soil enriched with nitrifying organisms.

Another phenomenon that has been observed is the remarkable bacteriostatic effect of potassium chlorate on the organisms that convert nitrite to nitrate (36). Small concentrations of chlorates, e.g., 10^{-6} M, have the power of preventing the development of Nitrobacter, whereas that of Nitrosomonas proceeds unchecked. The result is that when nitrogenous substances are nitrified in soil in the presence of small quantities of chlorates, nitrites but not nitrates accumulate. Potassium chlorate acts as a typical bacteriostatic agent. It does not poison or interfere with the bacterial oxidation of nitrite to nitrate, for with a bacteria-enriched soil the conversion of nitrite to nitrate proceeds at a constant rate uninfluenced by concentrations of chlorate which inhibit proliferation of the organisms involved. Chlorate bacteriostasis may be neutralized by the presence of nitrates, which appear to act specifically. Explanations of these phenomena are still lacking.

Inhibitors such as ethyl urethane gave qualitatively but not quantitatively the same results as those previously obtained by Meyerhof (42, 51, 60).

Later work showed the effects of various heavy metal ions on nitrification (32, 34), the effect of organic matter in immobilizing nitrate (34), the effect of amino acids, pH of the soil and the perfusate, narcotics and many other inhibitors on the oxidation of nitrite in soil (45, 51, 52). Still more recent work (51, 53) has shown the existence of a new form of nitrification whereby an organic nitrogenous compound, i.e., pyruvic oxime may yield nitrous acid directly without proceeding via ammonia.

Finally, in the present work, results will be cited which not only illustrate the many problems being faced today in the study of nitrification but also illustrate the specificity of action and stability of certain antibiotics and bacterial antagonists in soil. They describe in more detail the kinetics of soil nitrification and throw some light on the mechanism of ammonia oxidation.

PART I. NITRIFICATION OF AMMONIUM IONS AND OF AMINES

The work to be reported upon in this communication consists of a development of the investigations on soil nitrification using the modified soil perfusion

technique (4, 37). A preliminary statement giving the substance of some of the results to be described here has already been published (51).

The method of investigation is that of continuous perfusion in the dark at 21 C of a neutral solution of the substance under investigation through a column of 30 g air-dried crumbed soil (2 to 4 mm crumbs) under conditions of optimal aeration together with water saturation, but no water-logging. This technique of soil perfusion has already been fully described (37, 51). It may be mentioned that a battery of about 100 soil perfusion units kept in a thermostatically controlled room was employed.

Among the various conclusions previously reached, the following are of importance in connection with the present report:

1. When nitrification takes place in soil, the nitrifying organisms develop largely at the surfaces of the soil crumbs. During the initial perfusion of an air-dried soil this process may be of long duration, since the logarithmic growth phase is involved; but eventually a condition is reached when the sites of proliferation in the soil are saturated with the nitrifying organisms. The soil is now termed a saturated or enriched soil. Such a soil brings about a relatively rapid rate of nitrification. This rate remains constant during subsequent perfusions of the soil with ammonium chloride. The soil, in fact, behaves like an enzyme system, no proliferation of the relevant organisms taking place except presumably to replace the dead cells. The sites on the soil, at which Nitrosomonas[1] develops, are those where ammonium ions are held in base exchange. It seems possible that the adsorbed ammonium ions are preferentially taken up by the nitrifying organism.

2. A saturated or enriched soil that nitrifies an ammonium salt at a constant rate shows no initial lag period and will not nitrify organic nitrogen compounds such as methylamine, trimethylamine and glycine at constant rates. In fact, a lag period occurs showing that organisms must first develop that can convert these compounds into free ammonia which is subsequently nitrified. Pyruvic-oxime is very rapidly nitrified but it is now known that this process is accomplished by a heterotroph which has been isolated (51, 53).

Some of the factors influencing the kinetics of soil nitrification of ammonium ions and of amines will be discussed, and experiments showing the application of the Warburg manometric technique to the study of ammonia metabolism in enriched soils will be described.

Methods of Analysis

All analyses of samples from the soil perfusion apparatus have been made of either nitrate or nitrite accumulating as a result of the nitrifying processes. Estimations have been made colorimetrically.

Nitrate. A 1.0 ml sample of the soil perfusate is pipetted into a 25 ml volu-

[1] It has been found convenient, in this and subsequent papers, to refer to the soil organisms that convert ammonium ions to nitrite as Nitrosomonas and those that convert nitrite to nitrate as Nitrobacter. It is to be understood, however, that when these terms are used, there is no intention to eliminate other autotrophic organisms in the soil accomplishing these processes.

metric flask and evaporated to dryness in the presence of 0.2 ml 6% A.R. hydrogen peroxide (to oxidize any nitrites present to nitrate). On cooling, 1.0 ml phenoldisulphonic acid reagent is added and swirled around the flask. After standing for half an hour, 10.0 ml water and excess concentrated ammonia are added to each flask. On cooling the flasks, the volume is made up to the mark with ammonia and the intensity of the yellow color which develops is compared with that obtained from a standard potassium nitrate solution similarly treated. Accurate colorimetric assays are made using the Fisher Electrophotometer with filter B425. The extinction relative to that of a solution containing 70 μg nitrate-N is proportional to the logarithm of the concentration of nitrate-N in the sample (see fig. 1).

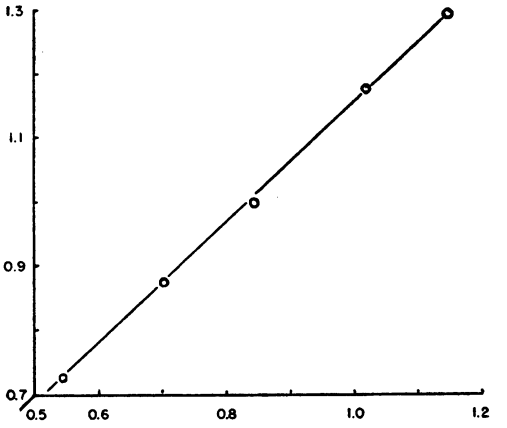


FIG. 1. Calibration curve for nitrate estimation

Ordinate: Relative extinction. (Relative extinction = 1 for 70 μg nitrate-N/ml) *Abscissa:* log (μg nitrate-N/ml).

Nitrite. This substance has also been estimated colorimetrically using the Fisher Electrophotometer with filter B525. The color is obtained with the Griess-Ilosvay reagent and compared with that obtained from a standard solution of M/4000 sodium nitrite. The samples obtained from the perfusion units are diluted accordingly. A direct proportionality exists between the concentration of nitrite present and the amount of color developed.

Results

Kinetics of soil nitrification of ammonium ions. Some details of the kinetics of the nitrification of ammonium ions in soil have already been discussed (37). It was found that the rate of nitrate formation, during the perfusion of ammonium chloride or sulphate, followed a sigmoid curve, which seemed to fit the equation:

$$\log \frac{y}{A-y} = K\,(t - t_1) \qquad [1]$$

where y = nitrate-N produced

A = asymptotic value approached by y

t = time

t_1 = time to $y = \frac{A}{2}$

K = constant

This equation had previously been discussed (43, 49) in connection with the process of nitrification.

More recent investigations, by ourselves, indicate that a simpler logarithmic relationship holds, i.e.,

$$\log y = kt + k' \qquad [2]$$

where k and k′ are constants.

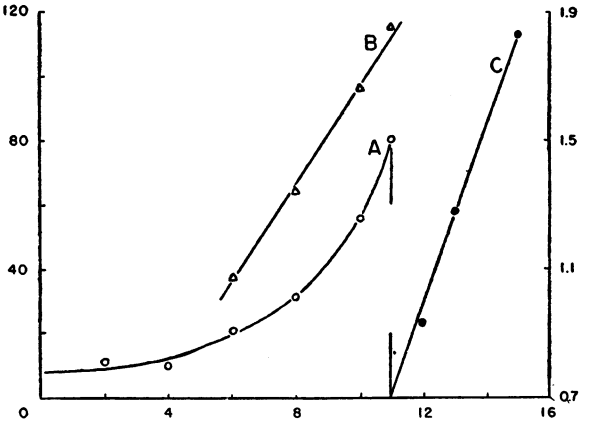


FIG. 2. Rates of nitrification of NH_4^+ during soil perfusion

A. Course of nitrate formation during initial perfusion of 0.01 M ammonium chloride through fresh soil.
B. Linear relationship between log y and t.
C. Course of nitrate formation during second soil perfusion of 0.01 M ammonium chloride.

Ordinate: Left, μg nitrate-N/ml (y). *Right,* $\log_{10}$ y. *Abscissa:* Time in days (t).

This experimental relationship helps to explain the fact that when a soil, that has been perfused with a solution of ammonium ions, is reperfused with a fresh solution of ammonium ions, the rate of nitrification during the second perfusion is constant and is almost the same as that at the end of the first perfusion. A typical set of results is shown in fig. 2. The simple exponential equation [2] gives the best correspondence with the facts. The rate should ultimately become a constant and should remain so when the soil is saturated with the organisms involved. On the other hand, if the sigmoid curve [1] is the better representation of the kinetic data, it is difficult to explain why there should be a decline in the rate of nitrification at the end of the first perfusion followed by a high and constant rate of nitrification during the second perfusion.

The exponential equation [2] which seems to hold in our soil perfusion work may be derived in the following way.

Let there be n_0 Nitrosomonas organisms present in the soil at any moment, of which n are non-viable. Then $n_0 - n = N$ are viable. Since the rate of proliferation may be expressed by the equation

$$\frac{dN}{dt} = KN$$

$$\log N = Kt + \text{constant.}$$

If the nitrifying capacity per cell remains constant, say p, then $\log pN = Kt + \text{constant}$. But the overall nitrifying activity, pN, of the viable cells equals $\frac{dy}{dt}$, where y is amount of nitrite produced. Since there is almost immediate conversion of nitrite into nitrate in a nitrifying soil, y is also equal to the amount of nitrate produced. Then

$$\log\left(\frac{dy}{dt}\right) = Kt + \text{constant}$$

$$\frac{dy}{dt} = K'e^{Kt}$$

$$y = K''e^{Kt} + \text{constant, where } K' \text{ and } K'' \text{ are constants.}$$

But when t is zero, y is also zero, so that

$$y = K''(e^{Kt} - 1)$$

Under our experimental conditions it is found that e^{Kt} is large compared with unity so that this expression approximates to

$$y = K''e^{Kt}$$

which may be rewritten as

$$\log_e y = Kt + \text{constant}$$

Granting no interfering factors, the amount of nitrate appearing in a perfusate should increase in a logarithmic manner with respect to time. The results given in fig. 2 show the validity of this conclusion. No results have been plotted where the nitrate concentration has been less than 20 μg/ml owing to the inaccuracy of the estimation in this region. It will be seen from fig. 2 that, on the second perfusion, the initial rate of nitrification is almost the same as that at the end of the first perfusion. This is entirely in accordance with the conclusion (37) that a condition of bacterial saturation of the surfaces of the soil crumbs arises when, in effect, the system behaves like a suspension of nonproliferating and yet actively metabolizing cells.

Effects of Changes of Hydrogen Ion Concentration on Soil Nitrification

1. *Buffering powers of soils.* The buffering power of a soil plays an important part in determining the rate of nitrification of ammonium salts. The optimum

rate of nitrification by Nitrosomonas in pure culture takes place at pH 8.5, the lower limit being at pH 4 (42). Since the oxidation of ammonium ions brings about the formation of nitric acid, it is obvious that the pH will fall progressively during nitrification unless there is good buffering. One of the most important factors controlling soil buffering is the amount of calcium carbonate present. A Cardiff soil which was neutral (pH 6.8) but which contained but little chalk showed a poor rate of nitrification of perfused ammonium chloride with little or no activity during the second perfusion. The addition of chalk (1 g to 30 g soil) improved the rate of nitrification strikingly and subsequent rates, on reperfusions, were constant and at a high level. Montreal garden soils, which have been used in almost all the work described in this paper, have a pH of 7.6 with a high

TABLE 1

Effects of the presence of sodium salts of organic acids and of glycerol and of glucose on the rate of nitrification of ammonium sulfate during perfusion through a Cardiff soil (pH 6.8, poorly buffered). 30 g of soil, 200 ml perfusing solution

Nitrate-N (μg/ml) Maximum = 140 μg/ml

PERFUSING SOLUTION	TIME IN HOURS						
	24.0	46.5	96.0	144.0	192.0	264.0	312.0
M/200 ammonium sulfate	11.5	23.0	45.0	56.0	68.5	92.0	129.0
M/200 $(NH_4)_2SO_4$ and M/200 sodium pyruvate	10.5	17.0	64.0	84.5	126.5	133.0	142.0
M/200 $(NH_4)_2SO_4$ and M/200 sodium acetate		25.0	59.5	85.5	126.0	138.5	135.0
M/200 $(NH_4)_2SO_4$ and M/200 sodium succinate	6.5	16.0	55.5	71.5	119.0	133.0	140.5
M/200 $(NH_4)_2SO_4$ and M/200 sodium bicarbonate	9.5	28.0	36.5	87.5	133.0	130.5	133.0
M/200 $(NH_4)_2SO_4$ and M/200 glycerol		23.0	17.5	36.5	74.0	94.5	115.0
M/200 $(NH_4)_2SO_4$ and M/200 glucose	12.0	12.5	27.5	26.5	39.0	51.0	80.5

chalk content; these show excellent rates of nitrification with subsequent rates, on reperfusion, constant and at the same high level.

2. *Effects of the addition of sodium bicarbonate to soil.* We (51) have shown that the addition of sodium bicarbonate (0.01 M) to a perfusate containing ammonium chloride (0.01 M) markedly improves the rate of nitrification in acid or slightly acid soils, but that the action is not so well sustained as when calcium carbonate is added.

3. *Effects of the addition of sodium salts of organic acids.* Admixture of sodium salts of organic acids such as pyruvic, succinic, or acetic with ammonium sulfate in the perfusing solution improves the rate of nitrification, but the improvement is almost identical with that effected by an equivalent concentration of sodium bicarbonate. Results illustrating this fact are shown in table 1. There seems to be little doubt that the accelerating action of the sodium salts of organic acids is due to their conversion, by soil heterotrophs, into sodium bicarbonate which by increasing the soil pH improves the rate of nitrification of ammonium ions.

4. *Effects of the addition of glycerol and of glucose.* As may be anticipated, the addition of glycerol or of glucose to a solution of ammonium sulfate does not increase the rate of nitrification.

The addition of glycerol has an initial depressing effect on the rate of nitrification, but in 8 days the rate is the same as that obtained with ammonium sulfate alone. The addition of glucose produces a much more sustained depression of the rate of nitrification, and, even after thirteen days perfusion, when with sodium salts of organic acids there has been 100% conversion of ammonia into nitrate and with glycerol 80%, there is with glucose only 57% conversion. Doubtless this depression is partly due to mobilization of nitrogen either from ammonia or nitrate by the heterotrophs assimilating the carbon of glucose and glycerol.

5. *Effects of the addition of amino acids.* If the conversion of an amino acid, say glycine, into nitrate by soil bacteria always requires the intermediate formation of the ammonium ion, it follows that the rate of nitrification of glycine should never exceed that of ammonium chloride under identical experimental conditions for equal quantities of nitrogen, chloride ions being relatively nontoxic (42). This however is not the case (51). With a soil of pH 6.5, the rate of nitrification of the glycine exceeds that of the ammonium chloride. Moreover, such a soil, after perfusion with glycine, becomes enriched with nitrifying organisms, subsequent perfusions with ammonium chloride showing relatively good rates of nitrification. The same soil perfused only with ammonium chloride fails to show such enrichment. A neutral or slightly alkaline soil, however, favors the nitrification of ammonium chloride rather than that of glycine, whereas an acid soil (pH 4.5) fails to nitrify either substance satisfactorily. The reason for the preferential nitrification of glycine over ammonium chloride in slightly acid soils (pH 6.5)—a phenomenon which must be of importance in connection with fertilizer problems—is easy to perceive.

When nitrification of one ammonium ion takes place, two hydrogen ions are iberated, thus:

$$NH_4^+ + 2O_2 \rightarrow 2H^+ + NO_3^- + H_2O$$

When glycine is nitrified, the following is the end result:

$$^+NH_3 \cdot CH_2 \cdot COO^- + 7(O) \rightarrow 2CO_2 + 2H_2O + H^+ + NO_3^-$$

In this case only one hydrogen ion is liberated per molecule of glycine, apart from the production of carbon dioxide, and hence the production of acidity will not be as great as during the nitrification of an ammonium salt of a mineral acid. Unless therefore the soil is well buffered, the fall of pH will be greater when ammonium chloride or sulfate is nitrified than when glycine, or any other amino acid is nitrified, for equivalent quantities of nitrogen. Doubtless the buffering action of the amino acid itself may play, initially, some role in maintaining the pH of the soil but this cannot be of long duration as the attack of heterotrophs on the amino acids in soil is very rapid and ammonia liberation quickly takes place.

Rates of nitrification of amino acids in soil

The rates of nitrate formation from such amino acids as glycine, alanine and glutamic acid on continuous perfusion through a well-buffered garden soil (pH 7.2) do not markedly differ from that from ammonium chloride when used at equivalent concentrations as previously shown (51). The results indicate that, in spite of the development of heterotrophic organisms that capture part of the available nitrogen, sufficient ammonia is produced to encourage the proliferation of nitrifying organisms at nearly optimal rates. Such a result proves that, under soil conditions, the presence of organic matter in the form of the simple amino acids quoted is no hindrance to the process of nitrification. The initial lag periods are of the same order as that obtained with ammonium chloride itself. The conclusion that amino acids *per se* (as well as such molecules as acetate, succinate, and glycerol) are not inhibitory to the development of the nitrifying organisms has recently (27) been confirmed.

A striking exception, however, presents itself with *dl*-methionine. This amino acid has a marked inhibitory action on soil nitrification (51). The inhibition will be considered in more detail later. Another amino acid whose presence in soil affects the rate of nitrification of ammonium salts is cysteine. Its depressing effect is in no way comparable with that of methionine, as results shown in fig. 3 indicate. In these experiments, sodium chlorate (0.001 M) was added to the perfusing solutions in order to inhibit the development of the Nitrobacter (36) and so make it easier to perceive the action of cysteine and methionine on the initial stage of nitrification of ammonia to nitrite. It will be observed that although neutral cysteine (M/100) retards the nitrification of ammonium chloride, the effect is that of a general slowing down as would be expected from a fall of pH. With *dl*-methionine (M/100), however, there is a long lag period during which there is neither nitrification of methionine itself, nor of the ammonium chloride, mixed with it. Eventually the lag ceases, doubtless when the methionine is completely decomposed, and then there is a rapid rate of nitrification both with methionine alone and with a mixture of methionine and ammonium chloride.

During cysteine perfusion through soil, free sulfate ions are formed and there is little doubt that the initial retardation of the rate of nitrification secured by cysteine is due to the fall of pH consequent upon the acidity produced on the liberation of sulfuric acid from cysteine oxidation. The retarding action of cysteine on nitrite oxidation in soil, may also be partly ascribed to the fall of pH due to sulfuric acid formation (45, 51, 52).

Effects of Organic Substances on Soil Nitrification of Ammonium Ions

1. *Urethanes.* Meyerhof (42) first showed that ethyl urethane inhibits the metabolism of isolated pure cultures of nitrifying organisms, giving 42% inhibition of the activity of Nitrosomonas at a concentration of 0.016 M and 4% inhibition Nitrobacter at a concentration of 0.011 M. This result was verified (37) and a higher sensitivity of nitrification to ethyl urethane in soil culture was shown. It was also demonstrated that the inhibitory effect of ethyl urethane is reversible. The presence of 0.01 M ethyl urethane greatly inhibits the nitrifica-

tion of 0.01 M ammonium chloride in garden soil (51), there being a complete suppression of nitrate formation for about twenty days. Thereafter the nitri-

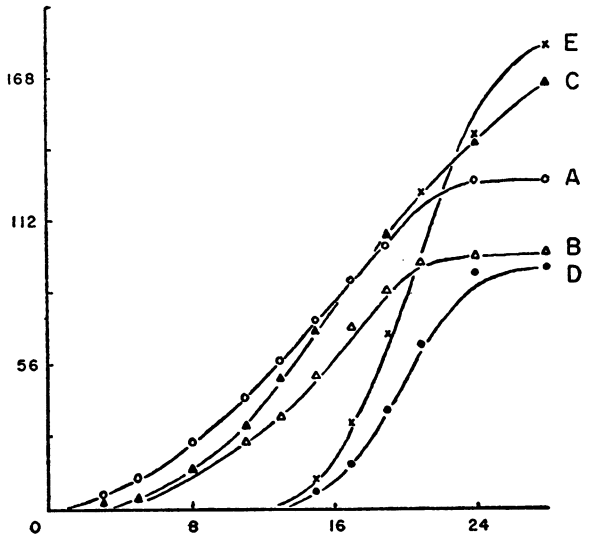


FIG. 3. The influence of methionine and cysteine on nitrification in fresh soil in the presence of 0.001 M sodium chlorate.

A. 0.01 M Ammonium chloride only.
B. 0.01 M Cysteine only.
C. 0.01 M Ammonium chloride and 0.01 M cysteine.
D. 0.01 M Methionine only.
E. 0.01 M Ammonium chloride and 0.01 M methionine.

Ordinate: μg nitrite-N/ml. *Abscissa:* Time in days.

TABLE 2

Effects of varying concentrations of ethyl urethane on nitrification of ammonium chloride (0.01 M). 30 g soil at 21 C

SUBSTANCES PRESENT IN PERFUSATE	TIME IN DAYS FOR NITRATE-N TO REACH 100 μg/ML	ESTIMATED LAG PERIOD IN DAYS
Ammonium chloride	13	0
Ammonium chloride and 0.0001 M ethyl urethane	14	1
Ammonium chloride and 0.00033 M ethyl urethane	16	3
Ammonium chloride and 0.001 M ethyl urethane	25	12
Ammonium chloride and 0.0033 M ethyl urethane	32	19

fication proceeds normally except that the nitrate nitrogen recovered accounts for 80% of the total nitrogen of the ammonium chloride and of the urethane. Urethane, itself, is ultimately metabolized by soil organisms.

The addition of different concentrations of ethyl urethane to a solution of ammonium chloride, that is being perfused through soil, brings about different lag periods, at the termination of which the rates of nitrate formation are very similar. Results given in table 2 show the different lag periods obtained with varying concentrations of ethyl urethane. A typical curve illustrating the course of nitrification of ammonium chloride in the presence of 0.01 M urethane is given in fig. 4.

The high sensitivity of the process of nitrification of ammonium ions to ethyl urethane, during soil perfusion, is not seen when the process is investigated manometrically using enriched soils over periods of short duration. The results

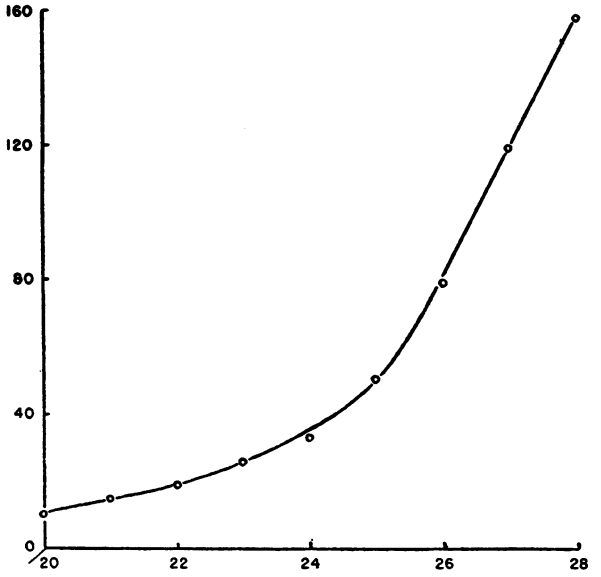


FIG. 4. The influence of ethyl urethane on nitrification in fresh soil. Perfusate of 0.01 M ammonium chloride and 0.1% ethyl urethane.
Ordinate: μg nitrate-N/ml. *Abscissa:* Time in days.

indicate that ethyl urethane impedes the assimilation of ammonium ions by Nitrosomonas, necessary for its growth.

Manometric investigation of ammonium ion oxidation by enriched soils. Soils that have been enriched or saturated with organisms attacking certain substrates may be conveniently investigated with the conventional Warburg manometric technique.

A soil known to be enriched with nitrifying organisms, through several perfusions of ammonium salts until complete nitrification has taken place, is washed with distilled water until the washings are free from nitrate and ammonium ions, and is pushed out of the soil tube on to a muslin cloth which is then wrapped around the soil This is suspended over a sink and the soil allowed to drain overnight (18 hours at 20 C). The soil is now squeezed gently to break up lumps and chopped with a spatula until it becomes a mass of small discrete crumbs rather

than a mud. The water content of this soil is about 50%. 1.5 g of the soil is weighed out and placed in a Warburg flask, care being taken to prevent soil entering the center well or the side arm. This is done by placing a small glass hood over the well and tilting so that the side arm is uppermost. 2.0 ml of an aqueous solution of the substance under investigation is added to the soil, which now becomes completely covered with fluid. Filter papers soaked in KOH solution are placed in the center well and the Warburg manometers are set up and shaken in the ordinary way. Experiments are carried out at 37 C, as, at this temperature, rates of oxygen uptake are appreciably higher than those at 21 C. There is no reason to suspect that the kinetics of oxidation by the soil organisms are adversely affected by the higher temperature. Attempts to store the damp soil for

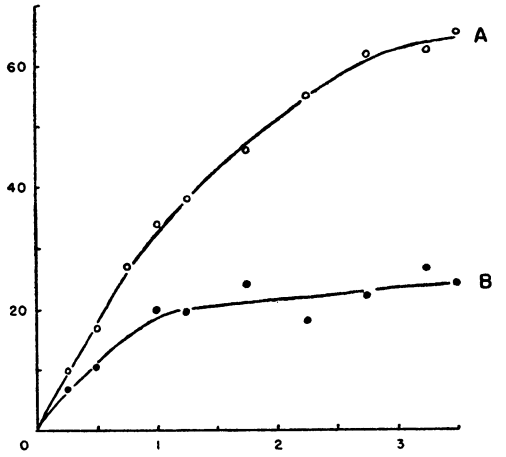


FIG. 5. The oxidation of ammonium chloride in enriched soils followed manometrically.
A. 1.0 ml. 0.002 M ammonium chloride added.
B. 0.5 ml. 0.002 M ammonium chloride added.
Ordinate: μl Extra oxygen uptake. *Abscissa:* Time in hours.

a longer period than that indicated have not proved profitable,—indeed the activity tends to fall off with time. A standard procedure has been to press the active soil in a clean cloth, to break it up as finely as possible, and to allow the finely divided soil to remain exposed to the air for half to one hour. Control manometric experiments are always carried out with soils suspended only in water. It has already been shown (20) that the respiration of a yeast suspension, in the presence of glucose, when added to soil is additive to the blank or water control of the soil itself.

Typical results showing the rates of oxygen uptake of washed enriched nitrifying soils in the presence of different quantities of ammonium chloride are shown in fig. 5. The difference between the total oxygen uptakes obtained with 1.0 ml M/500 ammonium chloride and 0.5 ml. M/500 ammonium chloride is 41.5 μl. For complete oxidation of ammonium chloride, viz.,

$$NH_4Cl + 2O_2 \rightarrow HCl + HNO_3 + H_2O,$$

the amount of oxygen required for the nitrification of 0.5 ml M/500 ammonium chloride would be $\frac{2 \times 22.4 \times 0.5 \times 1000}{500}$ μl = 44.8 μl. Therefore 92% of the theoretical value of oxygen was taken up. In general oxygen uptakes of the order of 85% of the theoretical values are obtained.

Effects of urethane on oxygen uptakes of enriched soils in the presence of ammonium ions. Data given in table 3 show the values of oxygen uptake by 1.5 g washed soil, enriched with nitrifying organisms (through several perfusions with 0.01 M NH_4Cl), alone, with ammonium chloride, and with ammonium chloride-ethyl urethane mixtures. It will be seen that ethyl urethane exercises no inhibition until the amount added to the Warburg flask is 1 ml M/100, its final concentration in this flask being M/275. The constant rate of oxygen uptake by an enriched soil respiring in the presence of ammonium chloride was found to fall to a lower level in the presence of the urethane. It is obvious from tables 2 and 3 that ethyl urethane, at a concentration ten times less than that at which a definite inhibition of the rate of oxygen uptake occurs, secures almost complete inhibition of the rate of proliferation of Nitrosomonas in soil.

TABLE 3

Oxygen uptakes in μl after 2.5 hours at 37 C by 1.5 g enriched nitrifying soil in the presence of ammonium chloride and ethyl urethane mixtures

SUBSTANCES ADDED TO SOIL IN THE WARBURG FLASK	OXYGEN UPTAKE IN μl	DIFFERENCES DUE TO NH_4Cl ADDITIONS
2 ml Water	44.0	
1 ml Water and 1 ml M/100 ammonium chloride	129.0	85.0
1 ml Water and 1 ml M/3000 urethane	44.5	
1 ml M/100 NH_4Cl and 1 ml M/3000 urethane	128.5	84.0
1 ml Water and 1 ml M/1000 urethane	34.5	
1 ml M/100 NH_4Cl and 1 ml M/1000 urethane	114.0	79.5
1 ml Water and 1 ml M/300 urethane	31.5	
1 ml M/100 NH_4Cl and 1 ml M/300 urethane	128.0	96.5
1 ml Water and 1 ml M/100 urethane	26.0	
1 ml M/100 NH_4Cl and 1 ml M/100 urethane	73.0	47.0

The inhibitory effect of ethyl urethane at concentrations of the order of M/200 on ammonia oxidation by enriched soils is also shown by N-methyl urethane. This substance at a final concentration of M/125 completely suppresses the oxygen uptake secured by an enriched nitrifying soil in the presence of M/125 ammonium chloride.

Later work has shown that ethyl urethane at the concentrations used exercises no inhibitions, such as those described, on other forms of soil metabolism that have been studied, including the metabolism of Nitrobacter. In these experiments

ethyl urethane causes an inhibition of the growth of Nitrosomonas at concentrations as small as M/5,000 and only secures 50% inhibition of ammonia oxidation at a concentration of M/275 when examined manometrically. These concentrations are small compared with those affecting metabolism in animal tissues or yeast. For example, a concentration of M/5 is required to inhibit rat brain cortex respiration by 50% (28), and a concentration of M/30 inhibits guinea pig brain respiration by only 20% and does not affect yeast respiration at all (55). This leads to the conclusion that ethyl urethane may have a high affinity for enzymes specifically concerned with ammonia metabolism.

A few experiments carried out with acetamide, show that this compound even at a concentration of M/125 has no inhibitory effect on the oxidation of ammonia

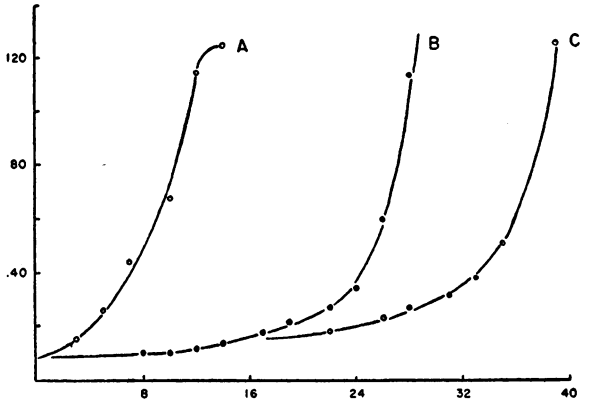


FIG. 6. The influence of guanidine carbonate on nitrification in fresh soil

A. 0.01 M Ammonium chloride.

B. 0.01 M Ammonium chloride and 0.0033 M guanidine carbonate at pH 7.0.

C. 0.0033 M Guanidine carbonate at pH 7.0.

Ordinate: μg nitrate-N/ml. *Abscissa:* Time in days.

by an enriched soil. It is clear that the $CONH_2$ group *per se* is not inhibitory so far as Nitrosomonas metabolism is concerned.

A study of the results obtained on perfusing mixtures of ammonium chloride and ethyl urethane through soils shows that nitrification takes place only when organisms have developed that break down the ethyl urethane.

2. *Guanidine.* Work with pure cultures of nitrifying organisms (42) and the soil perfusion technique (37) has shown that guanidine is a highly effective inhibitor of nitrification of ammonium ions. Results showing the effect of guanidine carbonate (M/300) on the nitrification of ammonium chloride (M/100) during soil perfusion are given in fig. 6. The effect of the guanidine is to produce a long lag, extending to about 20 days, before nitrification commences. The rate of nitrification then proceeds normally. It is interesting to note that nitrification of a mixture of guanidine and ammonium chloride commences before that of guanidine alone, i.e., at a point when there can be no appreciable breakdown of guanidine to ammonia. Either, therefore, the presence of ammonium ions lessens

the toxic action of guanidine or there occurs some adaptation of the nitrifying cells to guanidine whereby, in its presence, nitrification may take place. Such adaptation to a toxic substrate by nitrifying organisms will form the subject of discussion later, but a similar phenomenon is the adaptation of Nitrobacter to the toxic action of chlorate (36).

Both guanidine and N-methyl guanidine exercise, at a final concentration of M/275, marked inhibitions of the oxygen uptake secured by ammonium ions (M/275) in the presence of a washed nitrifying soil, enriched by repeated perfusion with ammonium chloride solution. Typical results are shown in table 4

TABLE 4

Oxygen uptake in μl after 2.5 hours at 37 C by 1.5 g enriched nitrifying soil in the presence of ammonium chloride and guanidine and methyl guanidine

SUBSTRATES ADDED	OXYGEN UPTAKE IN μl
2 m. Water	44
1 ml Water and 1 ml M/100 ammonium chloride	129
1 ml Water and 1 ml M/100 guanidine	20
1 ml M/100 NH_4Cl and 1 ml M/100 guanidine	21
1 ml Water and 1 ml M/100 N-methyl guanidine	30
1 ml M/100 NH_4Cl and 1 ml M/100 N-methyl guanidine	46

TABLE 5

Effects of thiourea and thiosinamine on the nitrification of ammonium chloride during soil perfusion

	μg NO_3^--N/ML PRODUCED AFTER 21 DAYS PERFUSION (MAXIMUM = 140)
0.01 M ammonium chloride	130
0.01 M ammonium chloride and 0.005 M thiourea	17
0.01 M ammonium chloride and 0.005 M thiosinamine	15

and indicate a toxicity of guanidine of over 80% which is superior to that of an equivalent concentration of ethyl urethane.

3. *Thiourea and thiosinamine.* Thiourea at a concentration of 0.00033 M will entirely prevent the nitrification of ammonium chloride in soil (51) even after a lapse of 16 days. There are no indications that thiourea will itself undergo nitrification. Thiosinamine (allyl-thiourea) is equally effective as an inhibitor of nitrification, as results in table 5 indicate. As previously shown (51) thiourea inhibits the oxygen uptake of an enriched nitrifying soil in the presence of ammonium chloride and no evidence of adaptation of nitrifying organisms to thiourea or thiosinamine has been secured.

Conceivably the toxic action of thiourea is explicable on the basis of possible combination at low concentrations with metallic ions, e.g., copper, which is said (32, 34) to be an important element in the development of nitrifying organisms or in the process of nitrification. This explanation does not seem at present, to

be entirely satisfactory for if the sole effect of thiourea is to immobilize metallic ions, it would be expected to have a higher toxicity to biological oxidations in animals tissues than is known to take place.

4. *Urea, arginine, creatine and glycine.* The influence of these substances on the respiration of a nitrifying soil has been investigated because of the relatively rapid production of nitrate during the perfusion of urea and glycine, and because arginine and creatine are guanidine derivatives and might act similarly to guanidine in depressing nitrification.

Results given in table 6 show that none of these substances has any inhibitory action on the oxygen uptake of an enriched nitrifying soil in the presence of ammonium chloride. The oxygen uptakes secured by glycine alone or ammonium chloride alone, on a nitrifying soil, are additive when the two substrates are mixed, pointing to no inhibition of ammonia oxidation by glycine at 0.0036 M. It has been found (27) however, that glycine at the higher concentration of

TABLE 6

Relative oxygen uptakes obtained with 1.5 g of washed enriched nitrifying soil at 37 C. The final concentration of all solutions was M/275. Oxygen uptakes were calculated relative to that of ammonium chloride on the same soil and all were corrected for water control

SUBSTRATE	AMMONIUM CHLORIDE ALONE	SUBSTRATE ALONE	MIXTURE OF AMMONIUM CHLORIDE AND SUBSTRATE
Glycine	100	33	129
Arginine	100	19	123
Creatine	100	4	110
Urea	100	132	127

0.013 to 0.046 M is inhibitory to nitrification by an isolated strain of Nitrosomonas. Arginine and creatine show no inhibitory effects on ammonia oxidation. Urea shows a high oxygen uptake, in the absence of added ammonium chloride, due to its rapid breakdown in soil by urease-containing organisms to ammonia, which is then oxidized. Clearly urea has no inhibitory effect on the process of nitrification in soil (see also 27).

5. *dl-Methionine and methionine sulfoxide.* Among the amino acids studied for their effects on soil nitrification only *dl*-methionine has so far been found to have a highly inhibitory effect on the nitrification of ammonium ions in soil. The depressing action of cysteine has already been commented upon; the nature of its effect differs considerably from that of methionine (45, 51, 52). In experiments where the rate of ammonium ion conversion into nitrite, during soil perfusion, was investigated by means of the addition of potassium chlorate which retards the development of Nitrobacter, it was found that *dl*-methionine (0.005 M) completely suppresses ammonia oxidation to nitrite for about 20 days at 21 C. Only when, eventually, the methionine begins itself to be broken down does nitrite appear either from ammonium ions or from molecules such as glycine. Results illustrating these facts have already been quoted (51). No adaptation of

the nitrifying organism to *dl*-methionine seems to take place; nitrification is delayed until the amino acid suffers decomposition.

Methionine sulfoxide appears to be about as effective as methionine in suppressing nitrification. Results illustrating its effect on nitrite formation from ammonia during perfusion through a soil in the presence of M/1000 sodium chlorate are shown in table 7.

The inhibitory effects of methionine and its sulfoxide take place at such low concentrations as to rule out the possibility that some decomposition to sulfuric acid, with consequent fall of pH, might be an explanation.

Turning to the consideration of other specific effects of methionine sulfoxide on metabolic processes, it is of importance to note the observations (65) on the action of this substance on the condensation of ammonia with glutamic acid to

TABLE 7

Percentage inhibitions of the rates of nitrification of 0.01 M ammonium chloride in the presence of 0.001 M sodium chlorate by varying concentrations of methionine sulfoxide when perfused through 30 g soil at 21 C

	PER CENT AMMONIUM ION NITRIFIED IN 15 DAYS	PER CENT INHIBITION
0.01 M ammonium chloride	60	
0.01 M ammonium chloride and 0.0001 M methionine sulfoxide	56	7
0.01 M ammonium chloride and 0.0003 M methionine sulfoxide	45	25
0.01 M ammonium chloride and 0.001 M methionine sulfoxide	34	43
0.01 M ammonium chloride and 0.003 M methionine sulfoxide	24	60

form glutamine in animal tissues. In this process methionine sulfoxide appears to act as a competitive inhibitor and at relatively low concentrations it will suppress glutamine synthesis.

With Nitrosomonas it is obvious that methionine sulfoxide is exerting a retarding action on a process leading to the proliferation of these cells. A satisfactory explanation of this effect of methionine sulfoxide would be at once available if the first process undergone by ammonium ions, in their metabolism by Nitrosomonas, is a condensation with glutamic acid, already present in the cells, to glutamine. The inhibitory action of methionine would be due, on this hypothesis, to a preliminary oxidation to methionine sulfoxide, which would be the effective inhibitor. This hypothesis of the first step in the assimilation of ammonia by Nitrosomonas being a condensation to glutamine is the subject of an investigation now being carried out and will be reported upon in due course.

6. *Bacterial inhibitors.* Many of the common inhibitors of aerobic bacterial metabolism will retard the process of nitrification in soil. Thus sodium azide and cyanide are effective inhibitors of nitrification and doubtless the inhibitory

action of quinhydrone is due to the well known bactericidal effects of quinones (37).

Sulfanilamide, sulfanilate, sulfamate. These substances were tested for their effects on the nitrification of ammonium chloride (0.01 M) during soil perfusion. Representative results are shown in fig. 7.

They show that sulfanilamide (0.005 M) exercises a very powerful inhibition of nitrate formation in soil, and sodium sulfanilate at the same concentration exerts a marked effect which is however weaker than that of sulfanilamide. In

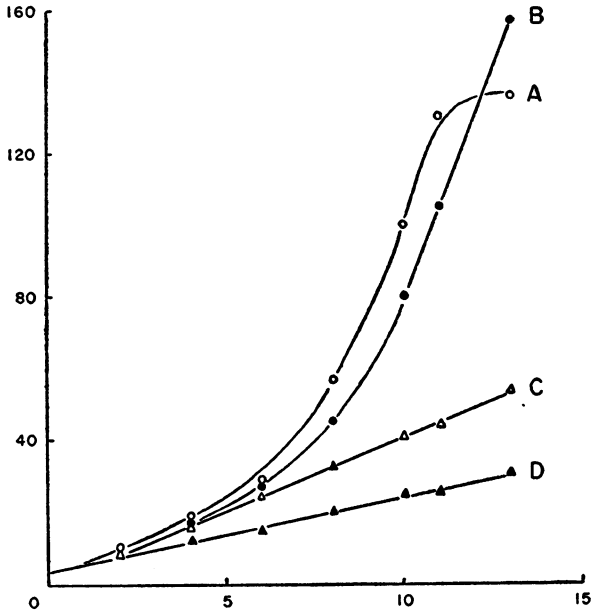


FIG. 7. The influence of some sulfur containing compounds on nitrification in fresh soil.
A. 0.01 M Ammonium chloride.
B. 0.005 M Ammonium chloride and 0.005 M ammonium sulfamate.
C. 0.01 M Ammonium chloride and 0.005 M sulfanilic acid (at pH 7).
D. 0.01 M Ammonium chloride and 0.005 M sulfanilamide.
Ordinate: μg nitrate-N/ml. *Abscissa:* Time in days.

both cases the rates of nitrification are constant. Ammonium sulfamate exercises no appreciable inhibitory action, and sulfamic acid does not seem to undergo any nitrification itself.

p-Aminosalicylic acid and p-aminobenzoic acid. *p*-Aminosalicylic acid (0.003 M) exerts a powerful inhibitory action on the nitrification of ammonium chloride perfused through soil. Definite inhibition can take place at a concentration as low as 0.0001 M. This effect is largely neutralized by *p*-aminobenzoic acid. These effects are shown in the figures quoted in table 8. *p*-Aminobenzoic acid, itself, has no inhibitory action on soil nitrification. Its apparent neutralizing action on the effect of *p*-aminosalicylic acid may be due to a true competition, such as occurs with sulfanilamide, or it may act by stimulating the development

of organisms to break down *p*-aminosalicylic acid. This is a matter for further investigation.

Sodium arsenite and sodium arsenate. In connection with work, which will be described in another paper, on arsenic metabolism in soil, experiments were carried out to determine the effects of sodium arsenite and sodium arsenate on

TABLE 8

Effects of p-aminosalicylic acid and p-aminobenzoic acid on nitrification in soil of ammonium ions

SUBSTRATE	μg/ml NITRATE-N FORMED IN 18 DAYS (MAXIMUM FOR AMMONIUM CHLORIDE = 140)
0.01 M ammonium chloride	129
0.01 M ammonium chloride and 0.003 M *p*-aminosalicylic acid	23
0.01 M ammonium chloride and 0.01 M *p*-aminobenzoic acid	153
0.01 M ammonium chloride, 0.01 M *p*-aminobenzoic acid, and 0.003 M *p*-aminosalicylic acid	120

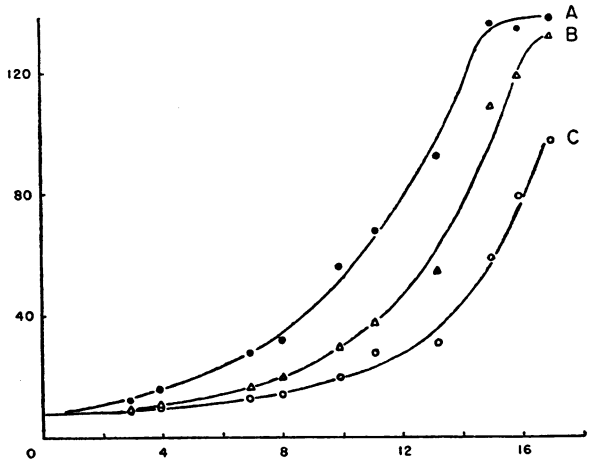


FIG. 8. The influence of sodium arsenate and arsenite on nitrification in fresh soil
A. 0.01 M Ammonium chloride.
B. 0.01 M Ammonium chloride and 0.0025 M sodium arsenate, at pH 7.
C. 0.01 M Ammonium chloride and 0.0025 M sodium arsenite at pH 7.
Ordinate: μg nitrate-N/ml. *Abscissa:* Time in days.

soil nitrification. Typical results showing the effects of the addition of M/400 sodium arsenite or sodium arsenate to a soil perfused with 0.01 M ammonium chloride are given in fig. 8.

The results indicate some toxicity of arsenate and a larger one of arsenite. With soils enriched with nitrifying organisms, sodium arsenate (M/400) showed no toxicity whereas sodium arsenite (M/400) exercised about 50% inhibition. The arsenite, however, is itself fully oxidized to arsenate within four days, so that the inhibitory effect of arsenite is only observable over this period.

Metabolism of amines in soil

Amines do not undergo direct nitrification in an enriched nitrifying soil (37) and a preliminary decomposition by heterotrophs must occur to produce ammonia which is subsequently nitrified.

Curves showing the rates of nitrification of methylamine and ethylamine in a fresh soil, and those obtained on reperfusion on the enriched soils, are shown in fig. 9. It will be seen that, after a preliminary lag on perfusion through a fresh soil, these amines are nitrified at rates similar to that of the ammonium ion. Subsequent perfusions show a much diminished lag period with almost constant rates of nitrification. This indicates enrichment of the soil with amine decomposing organisms as well as with the nitrifying bacteria.

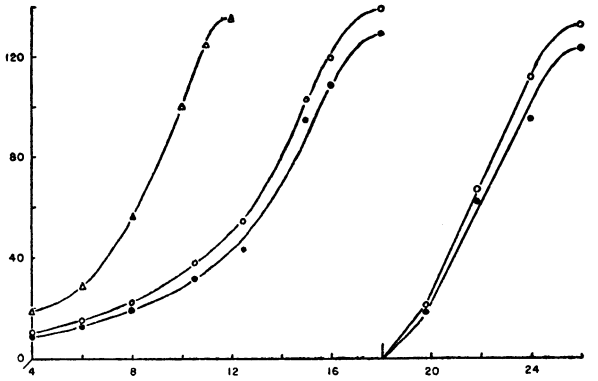


FIG. 9. The metabolism of simple amines in fresh soil

△ 0.01 M Ammonium chloride.
○ 0.01 M Ethylamine at pH 7, and result of reperfusion (18–26 days).
● 0.01 M Methylamine at pH 7, and result of reperfusion (18–26 days).
Ordinate: μg nitrate-N/ml. *Abscissa:* Time in days.

When these amines are tested as possible inhibitors of the nitrification of ammonium ions, it is found that methylamine has a decided inhibitory action at the concentrations used. This confirms the original observation (42) where an inhibition of 30% with M/200 methylamine was obtained in experiments on the respiration of proliferating Nitrosomonas. The results of a few experiments showing the inhibitory effects of methylamine on the rates of oxygen uptake by a washed enriched soil in the presence of M/275 ammonium chloride are shown in fig. 10. It will be noted that linear rates of oxygen uptake are obtained with each concentration of methylamine tested and this points to methylamine and ammonium ions coming to an equilibrium in the presence of the oxidizing mechanism, i.e., that the methylamine inhibition is of a competitive nature. This is seen very clearly from the results given in fig. 11 which shows that the percentage inhibition of nitrification by methylamine is a logarithmic function of its concentration. Results given in table 9 show that whereas methylamine shows little or no increase in oxygen uptake when added to a soil enriched by pre-

vious perfusions with ethylamine, the addition of ethylamine itself results in a decided increase in soil respiration. The addition of ethanolamine shows only a small increase in oxygen uptake. The addition of methylamine to a soil

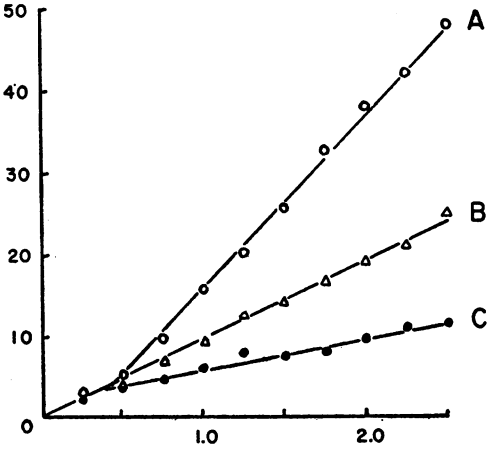


FIG. 10. The oxidation of ammonium chloride by enriched soil in the presence of methylamine.

A. 1.0 ml 0.01 M ammonium chloride; 1.0 ml water.
B. 1.0 ml 0.01 M ammonium chloride; 1.0 ml 0.033 M methylamine at pH 7.
C. 1.0 ml 0.01 M ammonium chloride; 1.0 ml 0.2 M methylamine at pH 7.

Ordinate: μl extra oxygen uptake. *Abscissa:* Time in hours.

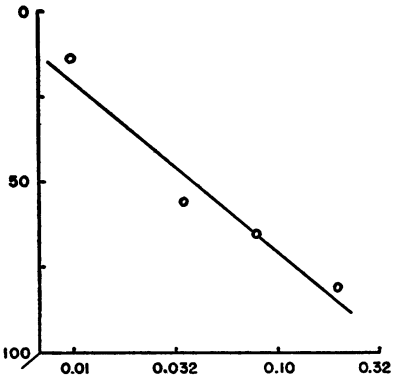


FIG. 11. The inhibition of oxidation of ammonium ions in an enriched soil by various concentrations of neutral methylamine.

Ordinate: Percentage inhibition. *Abscissa:* Molarity of methylamine.

already containing ethylamine brings about a suppression of the rate of oxygen uptake, due, doubtless, to competition with ammonium ions, as has been already pointed out, or with ethylamine itself. The effect of ethanolamine, on mixing with ethylamine, is to produce only a small inhibition of the rate of oxygen uptake.

The addition of ethylamine to a soil enriched only with nitrifying organisms (by previous perfusion with ammonium chloride) does not bring about any increase of the rate of oxygen uptake. This is to be expected for, as has been pointed out in the discussion of the kinetics of perfusion studies, the amine has first to be attacked by heterotrophic organisms. Representative results are shown in table 10. These results also show that ethylamine, in contrast to methylamine, does not inhibit ammonia oxidation at the concentrations cited.

Ethanolamine, on perfusion through soil, is rapidly nitrified. It is quite obviously attacked by heterotrophs that liberate ammonia which is subsequently

TABLE 9

Oxygen uptakes obtained with 1.5 g washed soil, previously perfused with 0.01 M ethylamine (neutral), either alone or with neutralized substrates. 4 hours at 37 C

SUBSTRATES ADDED TO WARBURG FLASK	OXYGEN UPTAKE IN μl
2 ml water	134
1 ml water and 1 ml 0.01 M ethylamine	396
1 ml water and 1 ml 0.01 M ethanolamine	185
1 ml 0.01 M ethylamine and 1 ml 0.01 M ethanolamine	383
1 ml water and 1 ml 0.01 M methylamine	125
1 ml 0.01 M methylamine and 1 ml 0.01 M ethylamine	192

TABLE 10

Oxygen uptakes alone and with substrates by 1.5 g washed soil previously perfused with 0.01 M ammonium chloride; 3 hours at 37 C

SUBSTRATES ADDED TO WARBURG FLASK	OXYGEN UPTAKE IN μl
2 ml water	90
1 ml water and 1 ml 0.01 M ammonium chloride	164
1 ml water and 1 ml 0.01 M ethylamine	95
1 ml 0.01 M ammonium chloride and 1 ml 0.01 M ethylamine	182

oxidized by nitrifying organisms. The kinetics of the nitrification of ethanolamine indicate that ammonia is an intermediate step because a soil, enriched with ethanolamine oxidizing organisms oxidizes ammonium chloride at a constant rate without an initial lag. A few manometric experiments were carried out with soils enriched by previous perfusion with ethanolamine oxidizing organisms and typical results are shown in table 11. It will be seen that the addition of ethanolamine secures a large increase in the rate of oxygen uptake. The addition of ethylamine shows no increase in the respiratory rate of the soil and ethylamine only reduces the rate of oxygen uptake due to ethanolamine by about 20%.

The results given in tables 9 and 11 show that the organisms that develop in response to the perfusion of ethylamine can attack this substance but only feebly oxidize ethanolamine, whereas those that develop in response to the perfusion of ethanolamine can attack this amine but they cannot oxidize ethylamine.

Discussion

It is of interest to list the effects on ammonia oxidation of the various metabolites that have been investigated. The results were obtained by studying the rates of oxygen uptake shown by soils enriched with nitrifying organisms through previous perfusions with solutions of ammonium chloride. Such a list is shown in table 12 where + indicates that inhibition takes place.

TABLE 11

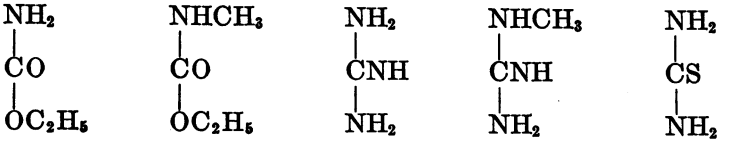
Oxygen uptakes either alone or with substrates at pH 7 by 1.5 g washed soil, previously perfused with 0.01 M ethanolamine (neutral); 2.5 hours at 37 C

SUBSTRATES ADDED TO WARBURG FLASK	OXYGEN UPTAKE IN μl
2 m. water	222
1 ml water and 1 ml 0.02 M ethanolamine	481
1 ml water and 1 ml 0.02 M ethylamine	194
1 ml 0.02 M ethanolamine and 1 ml 0.02 M ethylamine	399

TABLE 12

SUBSTANCES INVESTIGATED	INHIBITION OF RESPIRATION OF ENRICHED SOIL IN THE PRESENCE OF NH_4Cl
Methylamine	+
Ethylamine	0
Ethanolamine	0
Ethyl urethane	+
N-Methyl urethane	+
Guanidine	+
N-Methyl guanidine	+
Arginine	0
Creatine	0
Glycine	0
Methionine sulfoxide	+
Urea	0
Acetamide	0
Thiourea	+

If we compare structures of the inhibitory urethanes, guanidines, and thiourea, i.e.,



it will be seen that the structure in common has the form $R \cdot NH \cdot C \cdot OR'$ or $R \cdot NH \cdot C \cdot NH_2$. On the other hand urea $NH_2 \cdot CO \cdot NH_2$ is without inhibitory

action, and both arginine $COOH \cdot CHNH_2 \cdot (CH_2)_3 \cdot NH \cdot \overset{\overset{NH}{\cdot\cdot}}{C} \cdot NH_2$ and creatine $COOH \cdot CH_2 \cdot NCH_3 \cdot \overset{\overset{NH}{\cdot\cdot}}{C} \cdot NH_2$ are without effect. The amide linkage *per se* is also ineffective. It is obviously desirable to explore the effects of a variety of other molecules with a structure common to the urethanes, guanidines, and thioureas to discover the precise chemical and physical factors that are involved in the suppression of ammonia oxidation by Nitrosomonas. It seems likely that the affinity of the N atom in the structure $R \cdot NH \cdot C=$ for the ammonia oxidizing enzyme is primarily involved.

The highly inhibitory effects of methionine and methionine sulfoxide seem to belong to a different category from that mentioned above, and may perhaps be best explained on the hypothesis of competition for the enzyme that brings about the condensation of ammonia and glutamic acid to glutamine.

The suppressing action of *p*-aminosalicylic acid on nitrification seems again to belong to a different class of inhibitions, for the effects of *p*-aminosalicylic acid are obviously much less specific than those already discussed. The reversing action of *p*-aminobenzoic acid indicates that *p*-aminosalicylic acid belongs to a class of inhibitors to which sulfanilamide belongs, or that, as has been mentioned in the text, the growth of organisms, which can rapidly break down *p*-aminosalicylic acid, is stimulated by the presence of *p*-aminobenzoic acid.

It may also be fitting to summarize the knowledge that has been gained from our perfusion studies and manometric studies with enriched soils concerning the important question of the influence of organic matter on the process of nitrification.

Firstly it has been found that when glycine and arginine are added to a soil enriched with ammonia oxidizing organisms, they show increased oxygen uptakes which are completely additive to that due to the ammonium chloride. They are not therefore inhibitors of ammonia oxidation by Nitrosomonas.

Secondly, consideration must be given to the behavior of soils that become enriched with a variety of organisms through the perfusion with nitrogenous molecules. The general picture of these nitrifications is that of an initial slow rate of nitrate production followed by a more rapid rate which remains constant and at about the same value as that obtained when ammonium chloride is perfused through a soil enriched with nitrifying organisms. The initial low rates of nitrate formation are due doubtless to the preferential utilization of liberated ammonia by developing heterotrophs. As soon as the rate of growth of the heterotrophs falls, liberated ammonia becomes available for the nitrifying organisms which now develop at a normal speed. This may occur at a time when analysis shows that there is a considerable quantity of organic matter still left in solution. The latter is gradually oxidized by the resting heterotrophic population to ammonia.

Thus it may be concluded that organic matter does not, itself, influence the rate of nitrification except in so far as its nitrogen is diverted initially to the growth of heterotrophs. This is confirmed by our investigations of the metabolism

in soil of amino acids, amines, urethane and the sodium salts of organic acids, and of the effects of these substances on the course of nitrification of ammonium salts. A similar conclusion has been drawn recently (34) in a brief review of the question.

On the other hand, as has been emphasized, many organic molecules, e.g., methionine, methionine sulfoxide, urethanes and guanidines, exercise large but specific effects on the process of nitrification. Investigation of these should throw light on the mechanism of ammonia metabolism in Nitrosomonas.

Another fact that emerges from these studies is that the combination of perfusion and manometric studies on enriched soils makes it possible to study the mutual effects of organic molecules on the metabolism of organisms that have developed in soil as a response to the presence of these molecules. A number of interesting results emerge, as for example the fact that organisms that develop in response to the presence of ethylamine cannot oxidize ethanolamine and those that develop in response to the presence of ethanolamine cannot oxidize ethylamine. It is clear that the technique lends itself to the study of the development of organisms having highly specific metabolic relationships to the substrates perfused.

Summary of Part I

1. The rate of nitrate formation on perfusing ammonium chloride or sulfate through fresh soil at 21 C under optimal aeration conditions obeys the relationship:

$$\log y = Kt + K'$$

where y = concentration of nitrate formed and K and K′ are constants. This equation may be derived on the assumption that nitrification, during soil perfusion, is due to the metabolism of proliferating organisms.

2. After several perfusions of a soil with ammonium ions, a condition of bacterial saturation of the soil develops, when the rate of nitrification becomes, and remains, a constant. Such a soil behaves essentially as a system of non-proliferating but actively metabolizing cells.

3. The effects of changes of hydrogen ion concentration and buffering conditions in soil on the kinetics of nitrification are discussed. It is shown that the addition of chalk, or bicarbonate, improves nitrification on a poorly buffered, or acid, soil. The presence of sodium salts of organic acids, such as acetic, pyruvic, or succinic, increases the rate of nitrification in such a soil by giving rise to bicarbonate. The presence of glucose, or glycerol, on the other hand does not enhance the rate of nitrification, and may diminish the rate and the yield of nitrate formed, by diverting nitrogen from the nitrifiers to the heterotrophs proliferating at the expense of easily assimilable carbon.

4. The rate of nitrification of an amino acid, such as glycine, may exceed that of an equivalent concentration of ammonium ions in soil at a slightly acid pH [6.5] owing to the fact that the fall in pH due to amino acid oxidation is smaller than that due to the oxidation of ammonium salts of mineral acids.

5. The presence of organic matter in the form of amino acids, with the exception of cysteine and methionine, is no hindrance to the process of nitrification of ammonium ions in soil. The small retarding action of cysteine is due to the fall in pH consequent on sulfuric acid formation following oxidation of the amino acid. The inhibitory action of *dl*-methionine far exceeds that of cysteine and is not explicable by a fall of pH.

6. Soils, enriched or saturated with nitrifying organisms, may be studied with the conventional Warburg manometric technique. It is shown that almost the theoretical quantity of oxygen for the oxidation of NH_4^+ to NO_3^-, is taken up by such a soil in the presence of ammonium ions. The effects of a variety of substances on the rate of nitrification of ammonium ions in soil have been investigated with both the perfusion and manometric techniques.

7. Ethyl urethane at low concentrations (M/1000) greatly inhibits nitrate formation from ammonium ions on perfusion through a fresh soil. It has relatively little effect at such concentrations on nitrate formation from ammonium ions in a soil saturated with nitrifying organisms when examined manometrically; but at a concentration of M/275, it inhibits by 50% the rate of ammonium ion oxidation. Ethyl urethane, at low concentrations, interferes with some aspect of ammonia metabolism essential for the development of Nitrosomonas.

N-Methyl urethane suppresses ammonium ion oxidation by saturated soil at a concentration of M/125. Acetamide is without effect at this concentration.

8. Guanidine at low concentrations inhibits nitrification of ammonium ions in soil. Some evidence has been obtained that the nitrifying organisms are capable of adapting themselves to the toxic action of guanidine. Both guanidine and N-methyl guanidine (M/275) markedly inhibit oxygen uptake by enriched nitrifying soil in the presence of ammonium ions.

9. Thiourea and thiosinamine are powerful inhibitors of the nitrification of ammonium ions in soil. There is no evidence of adaptation of the nitrifying organisms to these substances.

10. Methionine sulfoxide resembles *dl*-methionine in its inhibitory effects on the process of nitrification of ammonium ions. It is suggested in view of the highly inhibitory effects of these amino acids that an important step in the assimilation of ammonia by Nitrosomonas is its conversion to glutamine, a process known to be retarded by methionine sulfoxide. No adaptation of the nitrifying organisms to the toxic action of methionine or its sulfoxide seems to occur.

11. Sulfanilamide has a highly inhibitory effect on the proliferation of the nitrifying organisms. Sodium sulfanilate has a weaker effect, and ammonium sulfamate is without action. Sulfamic acid is not nitrified.

12. *p*-Aminosalicylic acid is highly inhibitory to soil nitrification, its retarding effect being largely neutralized by the presence of *p*-aminobenzoic acid.

13. Sodium arsenite (M/400) inhibits soil nitrification, whereas arsenate has relatively little effect. Arsenite is oxidized in soil to arsenate.

14. Amines (methylamine, ethylamine, ethanolamine) are nitrified in soil after preliminary conversion to ammonium ions. Methylamine inhibits nitrification of ammonium ions, the inhibition being a logarithmic function of the concentra-

tion. Organisms that develop in soil in response to the perfusion of ethylamine can oxidize this amine but can only feebly oxidize ethanolamine; organisms that develop in response to the perfusion of ethanolamine attack this amine but they cannot oxidize ethylamine.

PART II. NITRITE OXIDATION IN SOIL

Since the isolation of nitrifying organisms (69), there has been but little investigation of the process of nitrite oxidation in soil. The properties of proliferating Nitrobacter were investigated (42) and it was shown that this organism oxidized nitrite to nitrate almost quantitatively, a fact which was confirmed by manometric studies on the isolated growing cells. The action of a variety of organic molecules on the respiration of Nitrobacter was also investigated and the optimal substrate concentration determined. Subsequently, the study of the kinetics of nitrite oxidation in soil was left almost alone until the advent of the perfusion technique. With this method the oxidation of nitrite in soil was followed (37) and the fact that increasing concentrations gave rise to increasing lag periods before nitrite metabolism commenced was established. It was shown (36) that addition of potassium chlorate to a solution of ammonium chloride, perfusing through soil, resulted in the almost exclusive formation of nitrite, there being no apparent change in the amount of chlorate present. The bacteriostatic action of chlorate on Nitrobacter disappeared in time, there being an adaptation to chlorate. Furthermore, increase of nitrate concentration, by initial addition of nitrate ions, reduced the toxic effect of chlorate on Nitrobacter. It was clear that chlorate, at the low concentrations that inhibited proliferation of Nitrobacter, had little effect on the oxidation of nitrite by these cells; for at such concentrations the formation of nitrate from nitrite by a soil enriched with nitrite oxidizing organisms was not impaired.

Field experiments (44), carried out with chlorate administration at the rate of 500 lbs per acre, showed a rather general bacteriostatic action of this substance. However 500 lbs/acre corresponds roughly to 0.04 g/sq inch and this to a concentration of M/150 chlorate in the top 6 inches of the soil assuming 50% moisture content, whereas the previous results were obtained with M/10,000 chlorate or about 0.002 g chlorate in 200 ml perfusate. Higher concentrations of chlorate exert marked inhibitory effects on Nitrobacter metabolism and the field results quoted are not unexpected.

A more detailed study of the kinetics of the proliferation of Nitrobacter, and of its oxidizing action on nitrite in soil has now been carried out and the details will be given in the following pages. Data will also be given to show the effects of a number of specific inhibitors, including nitrourea and chloromycetin, on the process of nitrite oxidation in the soil.

Methods. Estimations were made of the rates of appearance or disappearance of nitrite in the perfusion fluid. Nitrite was estimated as described in Part I.

Results

Metabolism of sodium nitrite in soil. When nitrite is perfused through soil under aerobic conditions and in the absence of added organic matter, it disap-

pears while giving rise to an equivalent concentration of nitrate. A typical result, wherein 97% of the theoretical quantity of nitrate resulted, is given in table 13. A correction has been applied for the normal amount of nitrate nitrogen present in perfusates after 15 days.

The oxidation of nitrite to nitrate is accomplished, in these perfusion experiments, by biological means. This follows from the facts that, at high initial concentration of nitrite, no change of concentration can be detected for very lengthy periods, and that the presence of chlorate at low concentrations will almost completely suppress the oxidation of nitrite in soil.

TABLE 13

Conversion of nitrite into nitrate during soil perfusion. Substrate perfused over 30 g soil at 21 C for 15 days until no nitrite left

SUBSTRATE	NITRITE-NITROGEN ADDED	NITRATE-NITROGEN APPEARED
Water	Nil	14 μg/ml
M/100 Sodium nitrite	140 μg/ml	150 μg/ml

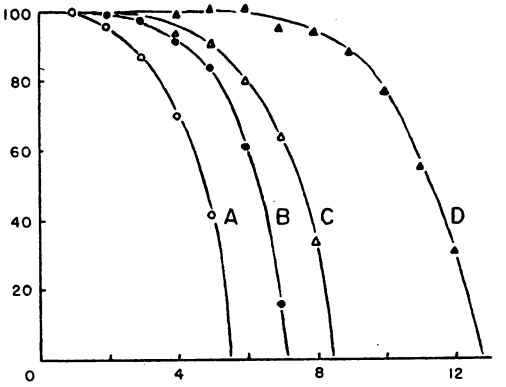


FIG. 12. The metabolism of sodium nitrite at various concentrations in fresh soil. A, 0.0025 M; B, 0.005 M; C, 0.01 M; D, 0.02 M.
Ordinate: Percentage nitrite remaining. *Abscissa:* Time in days.

Variation of rates of nitrite oxidation in soil with concentration. In all experiments using sodium nitrite solution as the perfusing fluid, a column of 30 g air-dried, crumbed and sieved, soil (2 to 4 mm crumbs) is used, the volume of perfusing fluid being 200 ml. Temperature is maintained at 21 C and experiments are run in the dark. The pH of the perfusing fluid is always 7.2 except when otherwise stated.

When sodium nitrite is perfused through a freshly dried crumbed soil, a lag period occurs which is followed by a rapid disappearance of the nitrite. The extent of the lag period increases with the initial concentration of the nitrite.

Results showing the variation of the rates of disappearance of nitrite with time are given in fig. 12. A family of exponential curves is obtained, each of which is characterized by initial delays which vary, in these perfusion experiments,

from one day, with an initial concentration of M/400 sodium nitrite, to six days with M/50 in the perfusate.

On plotting these curves logarithmically, i.e., log c (where c is the amount of nitrite oxidized) against time, a series of parallel straight lines is obtained, as shown in fig. 13.

Interpretation of the Kinetics of Nitrite Metabolism in Soil

It will be useful, at this stage, to give an analysis which will help in the understanding of the significance of the curves given in figs. 12 and 13.

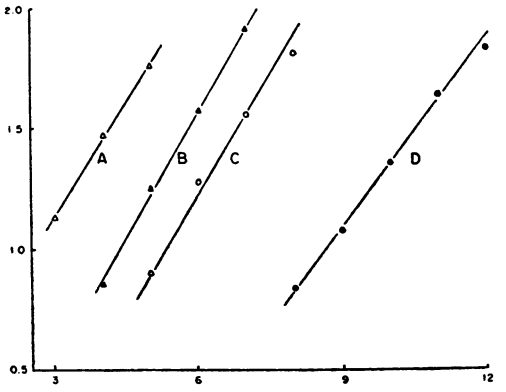


FIG. 13. The logarithmic relationship between the amount of nitrite oxidized and time. A, 0.0025 M; B, 0.005 M; C, 0.01 M; D, 0.02 M.
Ordinate: log c. *Abscissa:* Time in days.

Let N be the number of viable organisms in the soil at any time, capable of nitrite metabolism. Then the proliferation rate is given by the expression:

$$\frac{dN}{dt} = KN, \text{ where } K = \text{proliferation constant.}$$

Integration gives:

$$\log \frac{N}{N_0} = Kt$$

where N_0 is the number of viable organisms at zero time and N is the number at time t.

Let p = mean metabolic activity per viable cell, so that
pN = rate of metabolism for N viable cells.

If p remains constant during the proliferation period

then $\log \frac{pN}{pN_0} = Kt$

and $\log pN = Kt + \log pN_0$

If x = metabolic rate at time t, then $x = pN$ and $\log x = Kt + \log pN_0$

Now the experimental time T is made up of two components, t and L;

t = time of proliferation of N_0 organisms to N, and

L = lag period, during which no proliferation takes place and which, for a reason to be discussed below, is necessary before the nitrite-metabolizing organisms commence their growth.

Then $T = t + L$, so that

$$\begin{aligned} \log x &= K(T - L) + \log pN_0 \\ &= KT + \log pN_0 - KL \\ &= KT + A, \text{ where } A = \log pN_0 - KL \end{aligned} \quad [3]$$

Since the metabolic rate, or gradient of the curves in fig. 12, is a quantity which is difficult to estimate accurately, the above analysis has been carried a stage further. The expression given above

$$\log \left(\frac{pN}{pN_0}\right) = Kt$$

may be written as

$$pN = pN_0 e^{Kt}$$

Now pN is a measure of the metabolic activity and hence is a measure of the rate of change of the amount of nitrite oxidized, c. Therefore,

$$pN = \frac{dc}{dt}$$

$$\text{and } \frac{dc}{dt} = pN_0 e^{Kt}$$

$$c = \frac{pN_0}{K} e^{Kt} + K'$$

Since $c = 0$, when $t = 0$

$$c = \frac{pN_0}{K}(e^{Kt} - 1)$$

It has been found in practice that e^{Kt} is large compared with unity and the above expression approximates to

$$c = \frac{pN_0}{K} e^{Kt}$$

If c' = total amount of nitrite perfused

$$\frac{100c}{c'} = \text{percentage nitrite oxidized}$$

$$\log \left(\frac{100c}{c'}\right) = Kt + \text{constant}$$

which is the form followed in fig. 13.

The linear relationship between log x and T, as required by equation 3, is reflected in the results given in fig. 13. These results also show the important fact that K, the proliferation constant, remains unaltered for the family of curves and hence is independent of the initial concentration of sodium nitrite perfused through the soil. The constant A varies, however, according to this concentration.

Now it is clear that the spacing of the curves, as shown in fig. 13, is dependent upon A and therefore upon the two variables N_0 and L, the value of each of which may depend upon the initial concentration of sodium nitrite. The value of L may, in fact, be zero, but equation 3 will still yield a family of curves as shown in fig. 13, so long as N_0 varies with the initial concentration of nitrite present in the soil. The toxicity of nitrite to cell growth and enzymic mechanisms in the cell is a well known phenomenon (54, 56) and as a lethal substance it would be expected to reduce the number of viable organisms, including Nitrobacter, in the soil. It is known (42) that for Nitrobacter there is an optimal concentration of nitrite for growth. The increasing time lag with increasing concentration of nitrite in the perfusing fluid is presumably due, in the first instance, to the toxicity of nitrite to the nitrite-metabolizing organism and it would be expected, therefore, that N_0 would diminish as the initial concentration of nitrite is increased. The problem arises, however, as to whether the apparent proliferation constant, K, would remain unaltered for various initial concentrations of nitrite. The following brief analysis shows that this should not be the case.

We may expect that during the exposure of the organisms to the nitrite, the rate of dying off of the cells will be expressed by the relation

$$\frac{dN}{dt} = -K'N, \text{ where } K' \text{ is a constant.}$$

Combining this with the proliferation equation, we obtain

$$\frac{dN}{dt} = N(K - K')$$

and $\log x = (K - K')t + \log pN_0$

We may expect from the behavior of antiseptics at varying concentrations that K′ would be related to the concentration of the lethal substance according to the equation[2]

$$K' = K'' C^n$$

where K″ and n are constants and C = concentration.

Therefore, $$\log x = (K - K''C^n)t + \log pN_0 \qquad [4]$$

Now equation 4 would not give a series of parallel straight lines, as shown in fig. 13, as the expression $K - K''C^n$ is a function of the concentration of nitrite.

But since results in fig. 13 show that the relationship between log x and t is

[2] See Gay *et al.* "Agents of Disease and Host Resistance", C. C. Thomas (1935) p. 221; and "Topley and Wilson's Principles of Bacteriology and Immunity" by A. S. Wilson and A. A. Miles, 3d ed. (1946) Vol. 1, Chapt. 5. Arnold.

linear and the gradient is constant and independent of C, it follows that the relation given above cannot be operating during the proliferation period. The conclusion is that the nitrite metabolizing organisms, after exposure to the nitrite, have become resistant to its lethal effects. Two possibilities arise: (a) a mutant, or resistant strain immune to the toxic action of nitrite has developed or (b) the nitrite metabolizing cells have developed mechanisms which enable them to withstand the toxic action of nitrite at the concentrations under investigation, i.e., adaptation to the toxic substrate has taken place. The results given in figs. 12 and 13 are not sufficient to make it possible to judge between these two possibilities. Both hypotheses would yield the same mathematical results, for according to the first possibility the effect of the nitrite is to reduce N_0 (in equation 3) to the values of the numbers of mutant or resistant strains present, and according to the second possibility the effect of the nitrite is to increase the lag period, L, which would be the time required for the cells to become adapted to the toxic concentration of nitrite.

Bacterial Enrichment of the Soil

Results already secured (37, 51) have shown that during the nitrification of ammonium ions by soil, where the metabolite NH_4^+ is held in base exchange, a process of bacterial enrichment, and ultimately of saturation, takes place during continued perfusions through the soil. At the stage of bacterial saturation of the soil where all proliferation sites appear to be taken up, the soil accomplishes the oxidation of ammonium ions to nitrate at a constant rate with no initial time lag. The same phenomenon obtains during nitrite perfusion through the soil. When nitrite at a given concentration is perfused through soil until complete metabolism has occurred, the process being repeated several times with intervening washings, a condition of bacterial saturation ultimately obtains. At this stage the soil metabolizes nitrite at a constant rate with no initial time lag.

Experiments have been carried out to produce a number of soils, saturated with nitrite-oxidizing organisms by perfusion with sodium nitrite at different initial concentrations. The objective has been to discover how the metabolic activity of a saturated soil varies with the initial concentration of nitrite used for enrichment.

For these experiments soils were reperfused with sodium nitrite solution at a constant initial concentration as many as twenty times, extending over possibly three months, until it was certain that there was little or no further increase in activity of the soils as measured by their abilities to metabolize nitrite. Figure 14 shows a plot of the results obtained at seven different nitrite concentrations between M/25 and M/400. The results show that the metabolic activity of the saturated soil, expressed as the number of ml of M/400 sodium nitrite oxidized per hour per gram soil, varies in an approximately linear manner with the concentration of sodium nitrite with which the soil has been enriched with nitrite oxidizing organisms. It is concluded that the metabolic activity of a soil, at saturation, is a function (possibly linear) of the initial perfusing concentration of this substrate.

The question now arises as to whether the increase of metabolic activity of the saturated soil with increase of initial nitrite concentration is due (a) to an increase in the number of organisms, each having approximately the same metabolic activity per cell or (b) to an increase in the metabolic activity per cell, due to exposure to the higher initial concentration of substrate.

It is difficult to answer this question decisively owing to lack of a reliable method whereby the number of nitrite metabolizing cells in the soil can be estimated accurately. If, however, the assumption be made that bacterial saturation of a soil is due to occupancy of a limited number of sites on the soil crumb surfaces, and evidence for this has been given in the studies of nitrification of ammonium ions in soil (37), it would follow that soils, saturated with nitrite-metabolizing cells, would possess approximately the same number of such cells

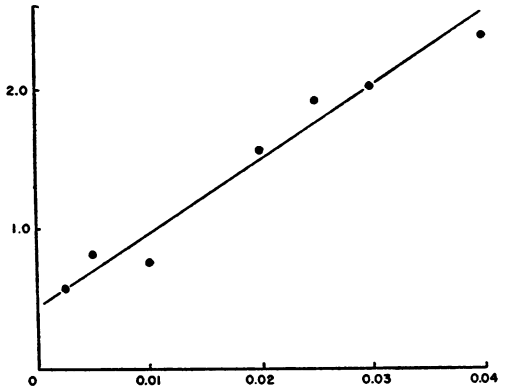


FIG. 14. The relationship between the metabolic activity of a saturated soil and the concentration of sodium nitrite used for enrichment.

Ordinate: ml 0.0025 M sodium nitrite oxidized per hour per gm soil. *Abscissa:* Molarity of nitrite perfused.

whatever the initial perfusing concentration of nitrite. It would be difficult, otherwise, to understand why a soil attains a constant metabolic rate whatever the number of perfusions and reperfusions of the substrate. It is to be remembered that a soil, after each perfusion and complete metabolism of substrate, is washed before it is reperfused so that toxic byproducts which might hinder the rate of growth are washed away. Moreover the cell growth takes place almost exclusively at the soil crumb surfaces, little or no cell development being seen in the perfusate. The simplest and most reasonable view is that cell growth ceases, or that a dynamic equilibrium between the proliferating and non-proliferating cells is set up, when the soil holds a limited and constant number of nitrite metabolizing organisms. If this view is correct it must be concluded that the metabolic activity per cell is increased in response to the exposure to a higher initial concentration of nitrite.

Metabolic activity per cell. It is to be noted that in the derivation of equation 3, it is assumed that p, which is the mean metabolic activity per viable cell, is a constant during proliferation. The fact that the experimental results are approxi-

mately in accordance with the equation gives support to this assumption. On the other hand, it has just been shown that the mean metabolic activity per cell is increased in response to the exposure to an increased initial concentration of nitrite. It is to be concluded, therefore, that this increase of metabolic activity per cell is present in the cells before proliferation commences. This would be expected (a) if a resistant strain, having the increased metabolic activity proliferates selectively or (b) if the change in metabolic rate takes place only during the lag period before proliferation commences.

Properties of Soils Saturated with Nitrite Metabolizing Cells

It was considered that further study of the properties of soils, saturated with the nitrite metabolizing cells, would throw more light on this problem and might help to settle the question as to whether a true adaptation takes place or whether selection of a resistant, or mutant, strain obtains. It has already been shown in a preceding section that adaptation of Nitrosomonas to a toxic substance such as guanidine can occur and there is no doubt that adaptation of Nitrobacter to chlorate takes place.

The problem of adaptation of microorganisms to toxic substance has been extensively studied by Hinshelwood and his colleagues (26). To test the view that adaptation may occur during a lag period when growth does not take place, Hinshelwood has considered enzyme kinetics, growth rates, duration of lag periods, serial subculture and reversion, effects of various nutrients, pH, concentration of toxic agents, etc. He has been able to make several mathematical deductions which when evaluated have given a single expression for families of curves with considerable accuracy. On the other hand it is evident that a great many of the experimental facts may well be explained on a selection hypothesis, and in any case it is sure that, once cells have become better adapted than others to proliferate in a certain medium, selection must immediately exercise an important influence.

It would be of importance to the general problem of adaptation if it can be shown that, during the growth of nitrite oxidizing cells in soil, a process of adaptation to the toxic substrate takes place and that during the lag periods, earlier described, the adaptive mechanisms operate.

For experiment, soils were enriched and finally saturated with nitrite-oxidizing organisms by continued perfusion and reperfusion with four different initial concentrations of sodium nitrite. It has already been shown that it is reasonable to assume that for saturated soils the number of nitrite-oxidizing cells held by the soil is a constant and independent of the metabolic activity of the cells. This assumption is based on the constancy of rates of reaction after saturation is reached. Thus the soils saturated after perfusion with the four different concentrations would all have the same numbers of nitrite oxidizing cells but with varying metabolic activities. Concentrations of M/400, M/200, M/100, M/50 sodium nitrite solution were used to enrich the soils and their metabolic activities were measured. These saturated soils, giving constant rates of nitrite oxidation, were then perfused with concentrations of sodium nitrite different

from those used to bring about enrichment and final saturation. Thus a soil, enriched with a perfusing concentration of M/400 sodium nitrite, was perfused with a much higher concentration of nitrite, e.g., M/100 and the subsequent metabolic rate, at the first perfusion, was noted. It was then perfused again with a different initial concentration of nitrite and the subsequent metabolic rate at the second perfusion was noted. The objective was to discover whether the organisms, proliferating at a concentration of nitrite of say M/400, would be able to metabolize a higher concentration, say M/100, without any initial lag, although, as shown in figs. 12 and 13 the initial lags with these different initial concentrations of nitrite on a fresh soil vary widely.

The results are shown in table 14 and the following conclusions may be drawn from them.

TABLE 14

Rates of nitrite oxidation by saturated soils subsequently perfused with various concentrations of sodium nitrite

ENRICHING CONCENTRATION	RATE* AT THIS CONCENTRATION	FIRST SUBSEQUENT CONCENTRATION	RATE* AT THIS CONCENTRATION	SECOND SUBSEQUENT CONCENTRATION	RATE* AT THIS CONCENTRATION	DELAY** IN DAYS BEFORE OXIDATION COMMENCES
M/400	0.059	M/200	0.056	M/50	Lag	1
M/400	0.056	M/100	0.059	M/25	Lag	3
M/200	0.080	M/100	0.070	M/40	Lag	0.75
M/200	0.088	M/50	Lag	M/25	Lag	1.5
M/100	0.074	M/50	0.081	M/25	0.070	<1
M/100	0.066	M/200	0.055	M/40	0.069	<1
M/50	0.148	M/200	0.130	M/25	0.105	<1
M/50	0.136	M/100	0.117	M/25	0.110	<1

* Rate expressed as ml M $NaNO_2$ oxidized per hour per 30 g soil.

** The normal delay in the fresh soil, prior to any perfusions, with initial concentrations of M/25 to M/50 $NaNO_2$ is greater than 15 days.

(a) A soil enriched at a given initial concentration of sodium nitrite can metabolize any lower concentration of nitrite without any initial lag period and will do so at a rate almost identical with that with which it metabolizes the substrate at the enriching concentration.

(b) A soil enriched at a given initial concentration of sodium nitrite can metabolize concentrations of nitrite up to four times that used for enrichment with an unaltered rate, but higher concentrations are metabolized at lower rates or with initial lag periods.

(c) Whenever a lag occurs in the case of a saturated soil metabolizing nitrite at a concentration appreciably higher than the enriching concentration, the duration of the lag period is far smaller than that obtained when the higher concentration is perfused for the first time over a fresh soil. Moreover, the sum of the original lag period and the second one induced by the higher concentration of nitrite perfused through the saturated soil, is much less than that resulting from the perfusion of the higher concentration over a fresh soil. Indeed, the

usual result of perfusing a higher concentration of nitrite over a soil saturated at a lower one is to produce a small diminution of metabolic activity rather than a lag period.

These results are in harmony with the view that, as a result of an adaptive process taking place during the lag period, the nitrite oxidizing cell acquires an increased concentration of a nitrite-oxidizing enzyme system. The increase is such as to enable the soil to oxidize nitrite, both at the enriching, and at considerably higher, concentrations, at the same rate and without any appreciable initial delay. It is evident that, once the soil is saturated at a given concentration of nitrite, no increased metabolic activity is immediately shown by subsequent perfusions of nitrite at higher concentrations. Increased metabolic activity is only secured by the higher concentrations of nitrite when lengthy initial delays are obtained, as shown in figs. 12, 13 and 14.

The most satisfactory explanation would be that, during the lag period, a process of adaptation takes place in a nitrite-oxidizing cell whereby it acquires the ability to proliferate in the presence of the given concentration of sodium nitrite, at the same time acquiring an increased concentration of a nitrite-oxidizing enzyme system. This cell now proliferates, to the exclusion of less favorably endowed cells, until it saturates the soil.

It may be questioned whether the endowment of the cell with an increased ability to oxidize nitrite is, in itself, sufficient to explain its ability to proliferate ultimately in the presence of the higher concentrations of the substrate. It is difficult to see why this should be so, unless the increased energy received by the cell through its higher rate of oxidation of nitrite enables it to withstand the toxic action of higher concentrations of nitrite. For example, if the toxic action of nitrite were due to an inactivating action on some key constituent of the cell (say for instance, adenosine triphosphate), the increased rate of oxidation of nitrite might result in an increased rate of synthesis of this constituent, sufficient to compensate for its rate of immobilization by the nitrite and thus allow proliferation to take place normally.

An alternative explanation to adaptation, i.e., selection and growth of a resistant strain already present in the soil, suffers from the fact that the assumption would have to be made that many such strains exist normally in the soil, each capable of proliferation at different concentrations of nitrite and each possessing correspondingly different powers of oxidizing nitrite. There is no proof, of course, that this is not the case, but it is easier to understand the general phenomenon as a process of adaptation than to explain it on the basis of the normal existence in soil of a multitude of variously resistant strains. The facts presented here, however, do not make it possible to decide between the two points of view.

Effects of perfusion of a soil with ammonium ions on its ability to metabolize nitrite. As it is an accepted fact that, during the conversion in soil of ammonium ions into nitrite, organisms that convert ammonium ions into nitrite and organisms that convert nitrite into nitrate arise, it would follow that perfusions of a soil with ammonium ions should bring about its enrichment with nitrite oxi-

dizing organisms. This can be tested by perfusing a soil, first with ammonium ions until the maximum rate of nitrate formation is obtained, and then with sodium nitrite. There should result a considerable diminution of the lag period which is normally obtained when sodium nitrite is perfused over a fresh soil.

Typical results are given in fig. 15 where it will be seen that the normal lag period on perfusing M/100 $NaNO_2$ solution over a fresh soil is greatly reduced when this concentration of nitrite is perfused through a soil previously enriched with nitrifying organisms by perfusion of M/100 ammonium ions. These results give additional evidence that enrichment of a soil with Nitrosomonas, after treatment with ammonium ions, entails enrichment also with Nitrobacter.

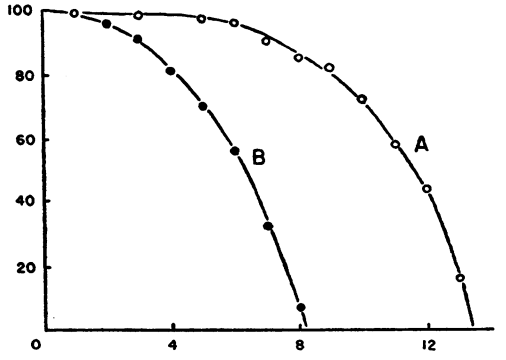


FIG. 15. The effect of prior perfusion of 0.01 M ammonium chloride on the metabolism of sodium nitrite in soil.

A. 0.01 M sodium nitrite perfused through fresh soil.

B. 0.01 M sodium nitrite perfused through soil previously treated with 0.01 M ammonium chloride.

Ordinate: Percentage nitrite remaining. *Abscissa:* Time in days.

The converse experiment, i.e., that of perfusing ammonium ions over a soil enriched with nitrite oxidizing organisms, indicates that such a soil shows no greater ability than a fresh soil to oxidize ammonium ions. It follows, as would be expected, that enrichment of a soil with Nitrobacter does not entail enrichment with Nitrosomonas.

Effects of dl-Ornithine and l-Arginine on Nitrite Metabolism in Soil

As we have previously shown (51), the presence of α-amino acids tends to retard the rate of disappearance of admixed nitrite (M/100), when perfused through soil, probably by giving rise, themselves, to nitrite. It was shown that the retardation by glycine, *dl*-alanine, sodium *dl*-aspartate or sodium *l*-glutamate, each at a concentration of M/100, is small. Cysteine produces a greater retardation, but *dl*-methionine has a highly inhibitory effect on nitrite utilization. After about ten days perfusion, however, nitrite metabolism takes place even in presence of *dl*-methionine which has not yet broken down to nitrite.

Arginine presents an anomalous case. When *l*-arginine (M/200) is mixed with

sodium nitrite (M/200), and the mixture is perfused through soil, the nitrite, instead of disappearing, gradually increases in quantity. After a preliminary lag, the nitrite concentration reaches a peak of nearly 200 μg N (as nitrite) per ml and subsequently decreases at a rapid rate. The whole process takes nearly 24 days. Typical results illustrative of the phenomenon are shown in fig. 16. The curves show the normal rate of metabolism of M/200 sodium nitrite on perfusion through 30 g soil, the small accumulation of nitrite and its rate of disappearance on perfusion of M/200 arginine alone, and the extraordinary rate of nitrite accumulation when a mixture of nitrite and arginine is perfused through the soil.

The unique behavior of *l*-arginine is in all probability due to the gradual liberation of urea which, by raising the local pH when hydrolyzed to ammonium

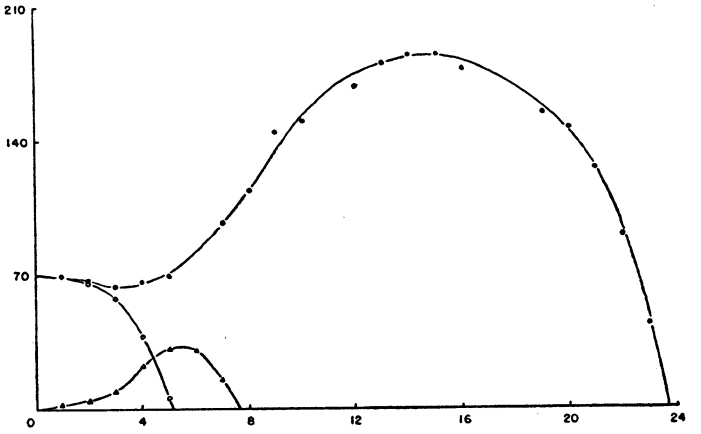


FIG. 16. The influence of arginine on nitrite oxidation in fresh soil

○ 0.005 M sodium nitrite perfused.
△ 0.005 M arginine (neutral) perfused.
● 0.005 M sodium nitrite and 0.005 M arginine perfused.
Ordinate: μg nitrite-N/ml. *Abscissa:* Time in days.

carbonate, not only facilitates the conversion of ammonium ions to nitrite, the optimum pH for which is 8.6 but, by contributing ammonium ions at a high pH, brings about an inhibition of nitrite oxidation. Thus, Meyerhof (42) has shown that M/1,000 ammonium ions at pH 9.4 inhibit the oxidation of nitrite by Nitrobacter by 55%.

This explanation may be tested by perfusing through soil mixtures of ornithine and nitrite, urea and nitrite, and ornithine, urea and nitrite, and comparing the rates of nitrite appearance with that obtained on perfusing a mixture of arginine and nitrite. The results of such an experiment are shown in table 15.

It will be seen that ornithine alone gives rise to little nitrite in the course of its metabolism in soil. In this matter, it resembles ammonium ions. Addition of sodium nitrite causes no accumulation of nitrite from the ornithine although it is probable that the production of nitrite and its disappearance are proceeding at the same rates (compare 51). When urea is perfused there is a definite production

of nitrite, probably because of the alkalinity and high concentration of ammonium ions developed on hydrolysis of urea to ammonium carbonate. When urea and sodium nitrite are perfused together the maximum net increase in nitrite concentration is not as great as with urea alone. Possibly the rate of formation of nitrite from the urea remains the same but metabolism of the added nitrite is taking place and this will have the effect of reducing the maximum value of nitrite formation. Simultaneous perfusion of urea and ornithine gives a maximum which is greater than the sum of the two individual maxima, but the addition of nitrite raises the maximum to the same extent as it raises that of urea alone.

When ornithine and urea are replaced by arginine the maximum is lowered and, in fact, is less than that given by urea alone. However, perfusion of arginine

TABLE 15

Maximum values of nitrite formation from various substances and mixtures of substances, all initially at pH 7.2 on perfusing through 30 g soil at 21 C. 0.005 M $NaNO_2$ = 70 μgN/ml

SUBSTANCES (0.005 M)	MAXIMUM VALUE OF NITRITE FOUND IN PERFUSING FLUID THROUGHOUT COURSE OF METABOLISM, EXPRESSED AS μg N PER ML
Arginine	33
Arginine + $NaNO_2$	186
Urea	43
Urea + $NaNO_2$	88
Ornithine*	4
Ornithine* + $NaNO_2$	No maximum value attained
Urea + ornithine*	76
Urea + ornithine* + $NaNO_2$	118
$NaNO_2$	No maximum value attained

* Ornithine concentration, 75 mg per 100 ml.

and nitrite simultaneously gives a very high maximum, much greater than any of the others, and is reached only after a period of about 15 days compared with 3 to 7 for the other maxima quoted. The greater efficacy of arginine in this respect may be attributable to a specific inhibitory effect of the compound itself, or of one of its intermediates, but it seems simpler to explain the result on the basis of change of pH. A slow liberation of urea (or ammonia and carbon dioxide) and ornithine would keep the pH high and thus maintain the observed effect over a longer period and so secure an increased maximum value of nitrite.

In all cases the accumulated nitrite eventually disappears as the pH drops and as adaptation of the nitrite oxidizing organisms to the high concentrations of nitrite with subsequent proliferation takes place.

Inhibitors of Nitrite Oxidation in the Soil

1. Potassium chlorate. The bacteriostatic action of chlorate at low concentrations (10^{-5} to 10^{-6} M) on the development of Nitrobacter has already been

described. It may be pointed out, however, that an inhibitive effect of chlorate at relatively high concentrations on the metabolism of nitrite in soil, enriched with nitrite oxidizing organisms, is observable. Thus chlorate at a concentration of 10^{-5} M will reduce this metabolic activity of a saturated soil by 15 to 20%, and a concentration as high as 10^{-3} M will completely suppress the activity. The results serve to indicate that chlorate is not without inhibitive action on the metabolism of the resting cells of Nitrobacter, but the main effect is obviously centered on proliferative processes which are susceptible to very small concentrations of the substance.

2. Chloromycetin. This antibiotic proves to be highly inhibitory to the metabolism of nitrite when it is perfused thrugh a fresh soil. The presence of

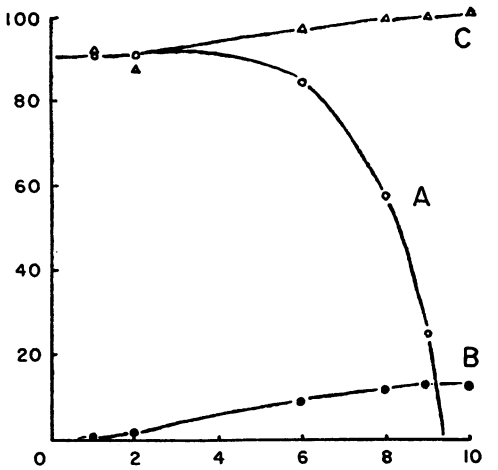


FIG. 17. The influence of chloromycetin on nitrite oxidation in fresh soil

A. 0.0025 M sodium nitrite.
B. Chloromycetin (25 mg percent).
C. 0.0025 M sodium nitrite and chloromycetin (25 mg percent).
Ordinate: Percentage nitrite remaining. *Abscissa:* Time in days.

chloromycetin at a concentration of 25 mg per cent (M/1,300) entirely prevents any disappearance of nitrite (M/400) within a period of ten days when perfused over 30 g of soil. In the absence of the chloromycetin, the nitrite disappears completely within this period. Typical results are shown in fig. 17. It should be noted that in the presence of chloromycetin alone there is a small but steady rate of appearance of nitrite due doubtless to the nitrification of residual ammonium ions in the soil.

Whether the inhibitive effect of chloromycetin on nitrite metabolism is connected with the possession of a nitro group in its structure is a matter for further investigation.

3. Nitrourea. This substance was chosen as a possible inhibitor of nitrite metabolism because of its possession of a nitro group which might enable it to compete with the nitrite ion for the enzyme involved.

Experiment shows that nitrourea at a concentration of M/300 completely inhibits the disappearance of M/100 nitrite on perfusion through soil when followed over a period of 16 days. Nitrourea, however, at this concentration does not inhibit the nitrification of ammonium ions, or nitrite formation from pyruvic-oxime, on perfusion through soil. These results are shown in table 16.

4. Nitromethane. This substance was also chosen as a possible inhibitor of nitrite metabolism because of the presence of a nitro group in its structure.

TABLE 16

The specific inhibitory effect of nitrourea on nitrite oxidation in the soil

SUBSTANCES IN PERFUSION FLUID	PERCENTAGE OF TOTAL PERFUSED NITROGEN* APPEARING AS NITRITE		
	3 days	10 days	16 days
M/100 sodium nitrite	100	5	
M/100 sodium nitrite and M/300 nitrourea	97	94	94
M/100 ammonium chloride and M/2000 potassium chlorate	2	22	48
M/100 ammonium chloride and M/2000 potassium chlorate and M/300 nitrourea	2	13	43
M/100 pyruvic-oxime (Na salt) and M/2000 potassium chlorate	75		
M/100 pyruvic-oxime (Na salt) and M/2000 potassium chlorate and M/300 nitrourea	82		

* Excluding the nitrogen of nitrourea.

TABLE 17

Effect of nitromethane on nitrite oxidation in the soil

SUBSTANCES PERFUSED	PERCENTAGE OF NITRITE-N REMAINING IN THE PERFUSATE						
	3 days	6 days	7 days	8 days	9 days	10 days	11 days
M/100 $NaNO_2$	99	80	56	25	0	0	0
M/100 $NaNO_2$ and M/100 nitromethane	99	95	89	83	68	49	6

Experiment shows that nitromethane (M/100) causes a relatively small increase in the normal lag period obtained when sodium nitrite (M/100) is perfused through a fresh soil. Typical results are shown in table 17. The effect of nitromethane must be considered small, especially when compared with the effects of other NO_2 containing molecules such as nitrourea and chloromycetin.

5. dl-Methionine. This amino acid exerts a powerful inhibitory effect on the nitrification of ammonium ions (51). It has a highly inhibitory effect also on the process of nitrite oxidation during perfusion through a fresh soil. Experimental results are shown in table 18.

The inhibitory effects of methionine on the activities of both Nitrosomonas and Nitrobacter make it seem likely that the glutamic-glutamine transformation may play an important role in the metabolism of these organisms (10, 39, 57, 65).

6. Thiourea. This substance at a concentration of M/100 proves to be an effective inhibitor of nitrite metabolism in a fresh soil. Table 18 displays a typical result.

7. Ethyl urethane. Ethyl urethane was chosen for investigation because of its highly inhibitory effect on the nitrification of ammonium ions on perfusion through soil. It proves to have no inhibitory effect at a concentration of M/100 on the proliferation of Nitrobacter, or on the rate of oxidation of nitrite perfused through a soil enriched with nitrite oxidizing organisms. Urethane (0.1%) inhibits the respiration of a growing culture of *Nitrobacter* by only 4 per cent (42).

TABLE 18

Effects of M/100 dl-methionine and M/100 thiourea on nitrite oxidation in soil

SUBSTANCE PERFUSED	PERCENTAGE NITRITE-N REMAINING IN PERFUSATE		
	4 days	8 days	17 days
M/100 sodium nitrite	93	33	0
M/100 sodium nitrite and M/100 *dl*-methionine	96	92	88
M/100 sodium nitrite and M/100 thiourea	94	93	84

TABLE 19

Effects of sodium arsenite on nitrite oxidation in soil

SUBSTANCE PERFUSED	PERCENTAGE NITRITE-N REMAINING IN PERFUSATE			
	4 days	5 days	6 days	7 days
M/400 sodium nitrite	90	75	49	4
M/400 sodium nitrite and M/400 sodium arsenite	94	95	97	95

8. Sodium arsenite. On perfusing sodium nitrite through soil in the presence of sodium arsenite, inhibition of nitrite oxidation takes place. Results given in table 19 illustrate this phenomenon. It will be seen that the presence of arsenite prevents disappearance of nitrite over a period of at least a week.

Manometric Experiments with Soils Enriched with Nitrite-Oxidizing Organisms

Soils that have been enriched or saturated with organisms metabolizing certain substrates may be conveniently investigated by the Warburg manometric techniques. Examples of this have been given in previous papers on the oxidation of ammonium ions by a soil saturated with Nitrosomonas (51). Application of these techniques makes it possible to study soils in a manner similar to that employed for animal or plant tissues and to explore metabolic events in the soil quantitatively.

Soils, enriched with ammonia oxidizing organisms, must be used in the form of aqueous suspensions for manometric investigations. This is necessary because air-drying of such soils causes a high degree of inactivation of these organisms.

This is not the case, however, with the nitrite-oxidizing organisms. Such organisms seem to be comparatively resistant to air-drying of the soil and will retain their activities in an air-dried soil for many weeks.

It is convenient to use such air-dried soils, enriched or saturated with nitrite-oxidizing organisms, for manometric studies, as far more soil than is possible when using soil suspensions may be employed in the manometric vessels. The technique adopted is that already described (20) for the study of the respiration of suspensions of yeast cells spread over soil crumbs.

For experiment a soil is enriched and finally saturated with nitrite-oxidizing organism as previously described. The soil is now removed from the perfusion unit, spread evenly on a glass surface and allowed to dry out in the air at room temperature. The air-dried soil is sieved, only particles of 1 to 2 mm being retained, and stored in a glass bottle in the refrigerator at 0 to 5 C. This soil retains its nitrite-oxidizing activity unimpaired for at least one week provided the drying out has been adequate.

About four grams of the air-dried soil crumbs are placed in a Warburg manometric vessel. Over this is spread carefully a small quantity of fluid, containing the substrate under investigation, sufficient to be adsorbed by the soil without any waterlogging taking place. Under such conditions, it has been shown that optimal aeration of the soil takes place and rates of oxygen uptake may be measured (so long as KOH is present to absorb released CO_2) without shaking of the Warburg manometers.

Oxygen uptake of a saturated soil. Experiments were carried out to measure the rate and amount of oxygen consumed by a soil saturated with nitrite oxidizing organisms when sodium nitrite was added. 3.75 g air-dried saturated soil are placed in each of a number of Warburg manometer vessels (containing KOH in the center cups). To one is added 1.5 ml water (allowing the fluid to spread as evenly as possible over the soil crumbs), to another a mixture of 1.0 ml water and 0.5 ml M/50 sodium nitrite solution is added, and to another a mixture of 0.5 ml water and 1.0 ml M/50 sodium nitrite solution. The vessels are attached to the appropriate manometers, placed in a water bath at 37 C (without shaking) for about 20 minutes, the taps closed, and the rates of oxgyen consumption measured. Typical results are shown in curves A and B, fig. 18, which record the changes of oxygen uptake with time, corrections having been made for the oxygen uptake of the water control.

It will be observed from curves A and B, that the velocities of oxygen uptake are almost constant and approximately the same until they cease through exhaustion of the substrate. The difference between the total oxygen uptakes (corrected for water control) for the oxidations of 0.5 ml M/50 $NaNO_2$ and 1.0 ml M/50 $NaNO_2$ (curves A and B) was 114.5 μl. This quantity is the amount of oxygen required to oxidize 0.5 ml M/50 $NaNO_2$. The theoretical quantity of oxygen required for this amount of nitrite according to the equation:

$$2NaNO_2 + O_2 = 2\ NaNO_3,$$

is $\frac{11200 \times 0.5}{50}\ \mu l = 112\ \mu l$

Thus, within experimental error, the theoretical consumption of oxygen is obtained when sodium nitrite is oxidized by a soil saturated with nitrite-oxidizing organisms.

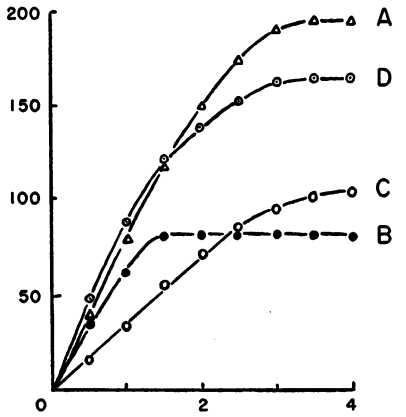


FIG. 18. The influence of sodium pyruvate on the oxidation of sodium nitrite in enriched soil studied manometrically.

A. 1.0 ml 0.02 M $NaNO_2$ + 0.5 ml water.
B. 0.5 ml 0.02 M $NaNO_2$ + 1.0 ml water.
C. 0.5 ml 0.02 M Na pyruvate + 1.0 ml water.
D. 0.5 ml 0.02 M $NaNO_2$ + 0.5 ml 0.02 M Na pyruvate + 0.5 ml water.

Ordinate: μl Extra oxygen uptake. *Abscissa:* Time in hours.

TABLE 20

Oxygen uptakes of a soil enriched with nitrite-oxidizing organisms. 3.75 g soil. 1.5 ml fluid containing substrate under investigation spread over the soil. 4 hours at 37 C

SUBSTRATE	OXYGEN UPTAKE*
1.5 ml M/75 sodium nitrite	238 μl
1.5 ml M/200 hydroxylamine (neutral)	10 μl
1.5 ml M/200 pyruvic-oxime (neutral)	26 μl
1.5 ml M/200 sodium pyruvate	71.5 μl

* Corrected for water control.

Oxygen uptakes in the presence of pyruvate, pyruvic-oxime and hydroxylamine

Experiments were carried out to see whether a soil enriched with nitrite-oxidizing organisms would bring about the oxidation of hydroxylamine, pyruvic-oxime or sodium pyruvate. Typical results are given in table 20. They show that, whereas sodium nitrite undergoes oxidation, neither hydroxylamine nor pyruvic-oxime brings about increased oxygen uptakes within experimental error. It is clear therefore that, under these experimental conditions, Nitrobacter does not secure oxidation of hydroxylamine or pyruvic-oxime.

With sodium pyruvate, however, there is an increased oxygen uptake of 71.5 μl. If oxidation of one mole pyruvate to one mole acetate were taking place, the

theoretical increased oxygen uptake would be 84 μl. In another experiment the increased oxygen uptake due to pyruvate was 90% of that calculated on the basis of conversion only to acetate. Sodium acetate, itself, secures no increase of oxygen uptake when added to a soil saturated with nitrite oxidizing organisms.

The conclusion would be that either Nitrobacter, or some other organism developing in the soil on continued perfusion of sodium nitrite, brings about the oxidation of sodium pyruvate, possibly to sodium acetate.

Curves C and D, fig. 18, show the rates of oxygen uptake secured by saturated soils in presence of sodium pyruvate and a mixture of sodium pyruvate and sodium nitrite. It appears that the oxidations of nitrite and of pyruvate by saturated soils proceed independently of each other, for the rate of oxygen uptake with the mixture is practically the sum of the individual rates. The result indicates that the presence of pyruvate does not diminish the ability of Nitrobacter to metabolize nitrite and this is in harmony with the observed fact that the addition of pyruvate has but little effect on their development in the perfusion experiments.

It is concluded, therefore, that the presence of organic matter such as pyruvate is not inhibitory to the respiration of Nitrobacter, a finding which is in harmony with the earlier results (42) on the action of glucose.

Summary of Part II

1. The kinetics of nitrite oxidation, on perfusion through soil at 21 C under optimal aeration conditions, have been investigated. When nitrite is perfused through a freshly dried, crumbed, soil, a lag period occurs that is followed by a rapid disappearance of the nitrite. The extent of the lag period increases with increase of the initial concentration of nitrite. The rate of nitrite oxidation obeys the relationship:

$$\log c = Kt + K'$$

where c is the amount of nitrite oxidized and K and K′ are constants. The derivation of this equation and the significance of the time lag are discussed.

2. The rate of proliferation of the nitrite oxidizing organisms is independent of the initial concentration of nitrite.

3. It is concluded, from kinetic evidence, that either (a) a mutant, or resistant strain immune to the toxic action develops with increased concentrations of nitrite, or (b) that nitrite metabolizing cells develop, by adaptation, mechanisms enabling them to withstand the toxic action of increased concentrations of nitrite.

4. Soils, on continued perfusion and reperfusion with nitrite, become enriched or saturated with nitrite oxidizing organisms. Such soils oxidize nitrite at constant rates.

5. The metabolic (nitrite oxidizing) activity of a soil, at saturation is a function (possibly linear) of the initial perfusing concentration of the nitrite. The conclusion is made that the metabolic activity per nitrite oxidizing cell is increased in response to exposure to higher initial concentration of nitrite.

6. Soils enriched at a given initial concentration of nitrite can metabolize any lower concentration of nitrite without any initial time lag and will do so at a rate almost identical with that at which it metabolizes the substrate at the enriching concentration. Such soils will also metabolize higher concentrations of nitrite, up to four times that used for enrichment, with an unaltered rate. Higher concentrations of nitrite than these are metabolized as a lower rate or with initial time lag. The time lag, however, is much smaller than that obtained when the high concentration of nitrite is perfused for the first time over a fresh soil. The results are in harmony with the view that, as a result of an adaptive process taking place during the lag period, the nitrite metabolizing cell acquires an increased concentration of a nitrite-oxidizing enzyme system and for some reason, not yet understood, is thereby enabled to proliferate in presence of otherwise toxic concentrations of nitrite.

7. During the perfusion of ammonium ions, the soil acquires the ability to oxidize nitrite without initial time lags indicating enrichment with the nitrite oxidizing organisms.

8. The presence of α-amino acids tends to retard the rate of disappearance of admixed nitrite when perfused through soil, probably by giving rise themselves to nitrite. The effects usually are small. *dl*-Methionine has, however, a highly inhibitory effect on nitrite utilization. Arginine is anomalous in that, in the presence of nitrite, the nitrite, instead of disappearing, increases markedly in quantity. It is shown that this phenomenon is probably due to hydrolysis of urea (liberated from the arginine) with formation of ammonia at a high pH. Under such conditions nitrite utilization in soil is inhibited.

9. The effect of chloromycetin, nitrourea and nitromethane on the oxidation of nitrite in soil has been investigated. Chloromycetin and nitrourea prevent proliferation of Nitrobacter at a concentration of M/1,000 and hence are of the same order of efficiency as potassium chlorate. Nitromethane has a definite though relatively small inhibitory effect on nitrite metabolism at a concentration of M/100.

Thiourea and sodium arsenite are inhibitory to nitrite oxidation in soil, but ethyl urethane (M/100) is without effect, in contrast to its marked action on ammonia oxidation in soil.

10. Soils saturated with nitrite oxidizing organisms may be investigated by the Warburg manometric technique. Using such soils, it is shown that the theoretical consumption of oxygen is obtained when sodium nitrite is oxidized by a soil saturated with nitrite-oxidizing organisms.

11. The presence of organic matter such as pyruvate is not inhibitory to the respiration of nitrite oxidizing organisms in soil. Soils saturated with such organisms cannot oxidize hydroxylamine or pyruvic-oxime.

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